DNA ENCODING APOPTOSIS-INDUCED EUCARYOTIC INITIATION FACTOR-5A AND DEOXYHYPUSINE SYNTHASE AND A METHOD FOR CONTROLLING APOPTOSIS IN ANIMALS AND HUMANS

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FIELD OF THE INVENTION

The present invention relates to polynucleotides which encode animal polypeptides that exhibit apoptosis-induced expression. The invention also relates to methods for modulating apoptosis in animals and humans. More particularly, the present invention relates to an apoptosis-induced eukaryotic initiation Factor-5A (eIF-5A) and an apoptosis-induced deoxyhypusine synthase (DHS) whose expressions are induced by the onset of apoptosis, and the use of the DHS gene and the eIF-5A gene, alone or in combination, as well as inhibitors of the DHS reaction, to modulate apoptosis in animals and humans.

BACKGROUND OF THE INVENTION

Apoptosis is the highly regulated process of programmed cell death that is essential for normal tissue development and homeostasis. It occurs in animals, humans and plants. In humans, for example, de-regulation of apoptotic processes contributes to a wide range of diseases, including disorders caused by loss of cells (e.g., neurodegenerative diseases, tissue damage from stroke or heart failure, and AIDS). Reed, J. C. and Tomaselli, K. J. (2000) *Curr. Opin. Biotech.*, 11, 586-592. In plants, the formation of water-conducting xylem vessels, a developmental process termed xylogenesis, entails programmed cell death that appears to be a plant counterpart of animal apoptosis. Specifically, apoptosis during xylogenesis features fragmentation of nuclear DNA, recruitment of cysteine proteases and the formation of apoptotic bodies, which are distinctive and characteristic features of animal apoptosis. Roberts, K. and McCann, M. C. (2000) *Current Opinion in Plant Biology*, 3, 517-522; Mittler R. and Lam

2

E. (1995) *Plant Physiol.*, **108**, 489-493; Ye, Z.-H. and Varner, J. E. (1996) *Plant Mol. Biol.*, **30**, 1233-1246; Minami, A. and Fukuda, H. (1995) *Plant Cell Physiol.*, **36**, 1599-1606; Beers, E. P. and Freeman, T. B. (1997) *Plant Physiol.*, **113**, 873-880; Iliev, I and Savidge R. (1999) *Phytochemistry*, **50**, 953-960; Yu, F.-X. and Chye, M.-L. (1999) *Plant J.*, **17**, 321-327; Groover, A. and Jones, A. M. (1999) *Plant Physiol.*, **119**, 375-384; Groover, A., DeWitt, N. G., Heidel, A. and Jones, A. M. (1997) *Protoplasma* **196**, 197-211. Inhibitors and elicitors of apoptosis have been cloned from a variety of species, but much of the apoptotic signaling pathway remains to be elucidated.

Cells undergoing apoptosis are identified by morphological changes which include cell shrinkage, condensation of chromatin, nuclear fragmentation, and the formation of apoptotic bodies (Reed and Tomaselli, 2000). One of the hallmarks of apoptotic cell death is the appearance of DNA fragmentation in multiples of 200 base pairs, which results from the activation of an endonuclease that cleaves the DNA between nucleosomes. Caspases, a family of proteases, are the main executors of the apoptotic program. Two major pathways to caspase induction include activation by molecules released from mitochondria, such as cytochrome c, and activation of death receptors by extracellular signals. Newmeyer, D. D., Farschon, D. M. and Reed, J. C. (1994) *Cell*, **79**, 353-364; O'Connor, L., Huang, D. C., O'Reilly, L. A. and Strasser, A. (2000) *Curr. Opin. Cell Biol.*, **24**, 49-52.

The unique amino acid, hypusine, is found in all examined eukaryotes and archaebacteria, but not in eubacteria, and eukaryotic initiation factor 5A (eIF-5A) is the only known hypusine-containing protein. Park, M. H. (1988) *J. Biol. Chem.*, **263**, 7447-7449; Schümann, H. and Klink, F. (1989) *System. Appl. Microbiol.*, **11**, 103-107; Bartig,

D., Scümann, H., and Klink, F. (1990) *System. Appl. Microbiol.*, **13**, 112-116; Gordon, E. D., Mora, R., Meredith, S. C., Lee, C. and Lindquist, S. L. (1987a) *J. Biol. Chem.*, **262**, 16585-16589. Active eIF-5A is formed in two post-translational steps: the first step is the formation of a deoxyhypusine residue by the transfer of the 4-aminobutyl moiety of spermidine to the ε-amino group of a specific lysine of the precursor eIF-5A catalyzed by deoxyhypusine synthase; the second step involves the hydroxylation of this 4-aminobutyl moiety by deoxyhypusine hydroxylase to form hypusine.

The amino acid sequence of eIF-5A is well conserved between species, and there is strict conservation of the amino acid sequence surrounding the hypusine residue in eIF-5A which suggests that this modification may be important for survival. Park, M. H., Wolff, E. C., and J. E. Folk (1993) *Biofactors*, **4**, 95-104. This assumption is further supported by the observation that inactivation of both of the eIF-5A's found to date in yeast, or inactivation of the deoxyhypusine synthase gene which catalyzes the first step in their activation, blocks cell division. Schnier, J. Schwelberger, H., Smit-McBride, Z., Kang, H. A., and Hershey, J. W. B. (1991) *Mol. Cell. Biol.*, **11**, 3105-3114; Sasaki, K, Abid, M. R. and Miyazaki, M. (1996) *FEBS Lett.*, **384**, 151-154; Park, M. H., Joe, Y. A. and Kang, K. R. (1998) *J. Biol. Chem.*, **273**, 1677-1683. However, depletion of eIF-5A protein in yeast resulted in only a small decrease in total protein synthesis suggesting that eIF-5A may be required for the translation of specific subsets of mRNA's rather than for protein global synthesis. Kang, H. A., Schwelberger, H. G., and Hershey, J. W. B. (1993) Effect of initiation factor eIF-5A depletion on cell proliferation and protein synthesis, in Tuite, M. (ed.), *Protein Synthesis and Targeting in Yeast*, NATO Series H. This view is supported by the recent finding that ligands which bind eIF-5A share highly conserved

motifs. Xu, A. and Chen, K. Y. (2001) *J. Biol. Chem.*, **276**, 2555-2561. The hypusine residue of modified eIF-5A was found to be essential for sequence-specific binding to RNA, and binding did not provide protection from ribonucleases. In addition, intracellular depletion of eIF-5A resulted in a significant accumulation of specific mRNAs in the nucleus, indicating that eIF-5A may be responsible for shuttling specific classes of mRNAs from the nucleus to the cytoplasm. Liu, Y. and Tartakoff, A. (1997) Supplement to *Molecular Biology of the Cell,* **8**, 426a. Abstract no. 2476, 37th American Society for Cell Biology Annual Meeting. The accumulation of eIF-5A at nuclear pore-associated intranuclear filaments and its interaction with a general nuclear export receptor further suggest that eIF-5A is a nucleocytoplasmic shuttle protein rather than a component of polysomes. Rosorius, O., Reichart, B., Kratzer, F., Heger, P., Dabauvalle, M.-C. and Hauber, J. (1999) *J. Cell Science,* **112**, 2369-2380.

The first cDNA for eIF-5A was cloned from human in 1989 by Smit-McBride *et al.*, and since then cDNA's or genes for eIF-5A have been cloned from various eukaryotes including yeast, rat, chick embryo, alfalfa, and tomato. Smit-McBride, Z., Dever, T. E., Hershey, J. W. B. and Merrick, W. C. (1989a) *J. Biol. Chem.*, **264**, 1578-1583; Schnier *et al.* (1991) (yeast); Sano, A. (1995) in Imahori, M. *et al.* (eds), *Polyamines, Basic and Clinical Aspects*, VNU Science Press, The Netherlands, 81-88 (rat); Rinaudo, M. S., and Park, M. H. (1992) *FASEB J.*, **6**, A453 (chick embryo); Pay, M. H., Wolff, E. C., Smit-McBride, Z., Hershey, J. W. B., and Folk, J. E. (1991) *Plant Mol. Biol.,* **17**, 927-929 (alfalfa); Wang, T. W., Lu, L., Wang, D., and Thompson J. E. (2001) *J. Biol. Chem.*, **276**, 17541-17549 (tomato).

Expression of eIF-5A mRNA has been explored in various human tissues and mammalian cell lines. For example, changes in eIF-5A expression have been observed in human fibroblast cells after addition of serum following serum deprivation. Pang, J. H. and Chen, K. Y. (1994) *J. Cell Physiol.*, **160**, 531-538. Age-related decreases in deoxyhypusine synthase activity and abundance of precursor eIF-5A have also been observed in senescing fibroblast cells, although the possibility that this reflects averaging of differential changes in isoforms was not determined. Chen, Z. P. and Chen, K. Y. (1997b) *J. Cell Physiol.*, **170**, 248-254.

Studies have shown that eIF-5A may be the cellular target of viral proteins such as the human immunodeficiency virus type 1 Rev protein and human T cell leukemia virus type 1 Rex protein. Ruhl, M., Himmelspach, M., Bahr, G. M., Himmerschmid, F., Jaksche, H., Wolff, B., Aschauer, H., Farrington, G. K., Probst, H., Bevec, D. and Hauber, J. (1993) *J. Cell Biol.*, **123**, 1309-1320; Katahira, J., Ishizaki, T., Sakai, H., Adachi, A., Yamamoto, K. and Shida, H. (1995) *J. Virol.*, **69**, 3125-3133. Preliminary studies indicate that eIF-5A may interact both with other RNA-binding proteins such as Rev, and also with Rev target RNA, suggesting that these viral proteins may recruit eIF-5A for viral RNA processing. Liu, Y. P., Nemeroff, M., Yan, Y. P. and Chen, K. Y. (1997) *Biol. Signals*, **6**, 166-174.

Spermidine analogs have been successfully used to inhibit deoxyhypusine synthase *in vitro*, as well as to inhibit the formation of hypusine *in vivo*, which is accompanied by an inhibition of protein synthesis and cell growth. Jakus, J., Wolff, E. C., Park, M. H., and Folk, J. E. (1993) *J. Biol. Chem.*, **268**, 13151-13159; Park, M. H., Wolff, E. C., Lee, Y. B., and Folk, J. E. (1994) *J. Biol. Chem.*, **269**, 27827-27832.

Polyamines themselves, in particular putrescine and spermidine, also appear to play important roles in cellular proliferation and differentiation. Tabor, C. W. and Tabor, H. (1984) *Annu. Rev. Biochem.*, **53**, 749-790; Pegg, A. E. (1988) *Cancer Res.*, **48**, 759-774. For example, yeast mutants in which the polyamine biosynthesis pathway has been blocked are unable to grow unless provided with exogenous polyamines. Cohn, M. S., Tabor, C. W., and Tabor, H. (1980) *J. Bacteriol.*, **134**, 208-213.

Polyamines have also been shown to protect cells from the induction of apoptosis. For example, apoptosis of thymocytes has been blocked by exposure to spermidine and spermine, the mechanism of which appears to be the prevention of endonuclease activation. Desiderio, M. A., Grassilli, E., Bellesia, E., Salomoni, P. and Franceschi, C. (1995) *Cell Growth Differ.*, **6**, 505-513; Brune, B., Hartzell, P., Nicotera, P. and Orrenius, S. (1991) *Exp. Cell Res.*, **195**, 323-329. In addition, exogenous polyamines have been shown to repress B cell receptor-mediated apoptosis as well as apoptosis in the unicellular parasite, *Trypanosoma cruzi*. Nitta, T., Igarashi, K., Yamashita, A., Yamamoto, M., and Yamamoto, N., (2001) *Exptl. Cell Res.*, **265**, 174-183; Piacenza, L., Peluffo, G. and Radi, R. (2001) *Proc. Natl. Acad. Sci.*, USA, **98**, 7301-7306. Low concentrations of spermine and spermidine have also been observed to reduce the number of nerve cells lost during normal development of newborn rats as well as protect the brain from neuronal damage during cerebral ischaemia. Gilad, G. M., Dornay, M. and Gilad, V. H. (1985) *Brain Res.*, **348**, 363-366; Gilad, G. M. and Gilad, V. H. (1991) *Exp. Neurol.*, **111**, 349-355. Polyamines also inhibit senescence, a form of programmed cell death, of plant tissues. Spermidine and putrescine have been shown to delay post-harvest senescence of carnation flowers and detached radish leaves. Wang, C. Y. and Baker, J.

E. (1980) *HortScience*, **15**, 805-806 (carnation flowers); Altman, A. (1982) *Physiol. Plant.*, **54**, 189-193 (detached radish leaves).

In other studies, however, induction of apoptosis has been observed in response to exogenous polyamines. For example, human breast cancer cell lines responded to a polyamine analogue by inducing apoptosis, and excess putrescine has been shown to induce apoptosis in DH23A cells. McCloskey, D. E., Casero, R. A., Jr., Woster, P. M. and Davidson, N. E. (1995) *Cancer Res.*, **55**, 3233-3236; Tome, M. E., Fiser, S. M., Payne, C. M. and E. W. Gerner (1997) *Biochem. J.*, **328**, 847-854.

Apoptosis is currently a field of intense study, and many techniques which may be useful in the control of the apoptotic process are being explored. A great deal of this research has been focused on inducing apoptosis in cancer models, but the prevention of unwanted apoptosis in disease processes is also under investigation. Activation of Jun kinase has been shown to be protective against nitric oxide-induced apoptosis in cardiac myocyte cells. Andreka, P., Zang, J., Dougherty, C., Slepak, T. I., Webster, K. A. and Bishopric, N. H. (2001) *Circul. Res.*, **88**, 305-312. Overexpression of anti-apoptotic proteins such as Bcl-2 in T and B lymphocytes has been shown to reduce apoptosis in lymphoid organs following cecal ligation and puncture, and decreased liver apoptosis has been observed in Bcl-2-overexpressing hepatocytes. Hotchkiss, R. S., Swanson, P. E., Knudson, C. M., Chang, K. C., Cobb, J. P., Osborne, D. F., Zollner, K. M., Buchman, T. G., Korsmeyer, S. J. and Karl, I. E. (1999) *J. Immunol.*, **162**, 4148-4156; Rodriguez, I., Matsuura, K., Khatib, K., Reed, J. C., Nagata, S. and Vassalli, P. (1996a) *J. Exp. Med.*, **183**, 1031-1036. Inhibitors of caspases have also been used to block Fas-mediated

apoptosis in the liver. Rodriguez, I., Matsuura, K., Ody, C., Nagata, S. and Vassalli, P. (1996b) *J. Exp. Med.,* **184**, 2067-2072.

Inhibition of caspase 1 has also been observed to slow the progression neurodegenerative disease in a mouse model of Huntington's disease. Ona, V. O., Li, M., Vonsattel, J. P., Andrews, L. I., Khan, S. Q., Chung, W. M., Frey, A. S., Menon, A. S., Li, X. J. and Stieg, P. E. *et al.* (1999) *Nature,* **399**, 263-267. Animal models have also been used to demonstrate the efficacy of peptidyl caspase inhibitors in stroke, myocardial infarction, sepsis, and amylotrophic lateral sclerosis. Endres, M., Namura, S., Shimizu-Sasamata, M., Waeber, C., Zhang, L., Gomez-Isla, T., Hyman, B. T. and Moskowitz, M. A. (1998) *J. Cereb. Blood Flow Metab.,* **18**, 238-247 (stroke); Wiessner, C., Sauer, D., Alaimo, D. and Allegrini, P. R. (2000) *Cell. Mol. Biol.,* **46**, 53-62 (stroke: caspase inhibitor z-VAD-fmk decreased cortical infarct by 45% after permanent middle cerebral artery occlusion in rat); Rabufetti, M., Sciorati, C., Taroxxo, G., Clementi, E., Manfredi, A. A. and Beltramo, M. (2000) *J. Neurosci.,* **20**, 4398-4404 (stroke: caspase inhibitor Ac-YVAD-cmk reduced infarct volume and decreased apoptosis, as measured by nucleosome quantitation, by approximately 50% after permanent middle cerebral artery occulusion in rats); Holly, T. A., Drincic, A., Byun, Y., Nakamura, S., Harris, K., Klocke, F. J. and Cryns, V. L. (1999) *J. Mol. Cell. Cardiol.,* **31**, 1709-1715 (myocardial infarction: systemic administration of caspase inhibitor YVAD-cmk reduced myocardial infarct size by 31% and reduced the number of apoptotic cells by approximately 70% in rabbits following coronary artery occlusion and reperfusion); Jaeschke, H., Fisher, M. A., Lawson, J. A., Simmons, C. A., Farhood, A. and Jones, D. A. (1998) *J. Immunol.,***160**, 3480-3486 (sepsis: caspase inhibitor z-VAD attenuated apoptosis by 81 to 88% and

prevented liver cell necrosis in mice experiencing endotoxin-induced liver damage); Li, M., Ona, V. O., Guegan, C., Chen, M., Jackson-Lewis, V., Andrews, L. J., Olszewski, A. J., Stieg, P. E., Lee, J. P., Predborski, S. and Friedlander, R. M. (2000) *Science*, **288**, 335-339 (amylotrophic lateral sclerosis). These studies indicate that apoptosis can be controlled to some degree by targeting the core components of the cell-death machinery. Reed, J. C. and Tomaselli, K. J. (2000).

However, methods for controlling apoptosis and for treating disease by controlling apoptosis are still a largely unexplored area. Failures in the precise regulation of apoptosis are believed to cause or exacerbate such diverse diseases and disorders as neurological/ neurodegerative disorders (e.g., Alzheimer's, Parkinson's, Huntington's, Amyotrophic Lateral Sclerosis (Lou Gehrig's), stroke, autoimmune disorders (e.g., rheumatoid arthritis, systemic lupus erythematosus (SLE), multiple sclerosis), Duchenne Muscular Dystrophy (DMD), motor neuron disorders, ischemia, chronic heart failure, infantile spinal muscular atrophy, cardiac arrest, renal failure, atopic dermatitis, sepsis and septic shock, AIDS, hepatitis, glaucoma, diabetes (type 1 and type 2), asthma, retinitis pigmentosa, osteoporosis, xenograft rejection, and burn injury. Thus, methods of controlling apoptosis are needed.

SUMMARY OF INVENTION

This invention is based, in part, on the discovery and cloning of a full length cDNA encoding a novel apoptosis-induced eIF-5A from rat corpus luteum. The nucleotide sequence and corresponding amino acid sequence are disclosed herein.

The invention is also based, in part, on the discovery and cloning of a partial length cDNA clone encoding a novel apoptosis-induced deoxyhypusine synthase from rat

corpus luteum. The nucleotide sequence and corresponding amino acid sequence are disclosed herein.

The invention provides an eIF-5A cDNA which is up-regulated immediately before the induction of apoptosis. This apoptosis-induced eIF-5A is a suitable target for regulation of apoptosis, including apoptosis underlying disease processes, since it acts in the post-transcriptional regulation of downstream effectors and transcription factors involved in the apoptotic pathway.

The present invention also provides methods for modulating or altering apoptosis, and thus controlling diseases attributable to the induction of apoptosis, by gene therapy. In one embodiment of the invention, apoptosis-induced eIF-5A nucleotide sequences of the invention, fragments thereof, or combinations of such fragments, are introduced into animal/human cells or tissue in reverse orientation to inhibit expression of the endogenous apoptosis-induced eIF-5A gene, thereby reducing the level of endogenous apoptosis-induced eIF-5A protein, and thereby reducing and/or preventing expression of the genes that mediate apoptosis.

In another embodiment of the invention, the apoptosis-induced DHS nucleotide sequence of the invention, fragments thereof, or combinations of such fragments, are introduced into animal/human cells or tissue in reverse orientation to inhibit expression of the endogenous apoptosis-induced DHS gene, and thereby reduce the level of endogenous apoptosis-induced DHS protein, and thereby reducing and/or preventing activation of apoptosis-induced eIF-5a and the ensuing expression of the genes that mediate apoptosis.

11

In yet another embodiment of the present invention, both the apoptosis-induced DHS sequence and the apoptosis-induced eIF-5A sequence can be used together to reduce the levels of endogenous apoptosis-induced DHS and eIF-5A proteins.

Using the methods of the invention, a vector, such as a viral vector, can be used to achieve transient expression of the apoptosis-induced eIF-5A nucleotide sequence, fragments thereof, and/ or combinations of such fragments, or the apoptosis-induced DHS nucleotide sequence, fragments thereof, or combinations of such fragments in the antisense orientation in animal/human cells and tissues.

In one embodiment, the present invention is directed to an isolated DNA molecule encoding apoptosis-induced eIF-5A, wherein the DNA molecule hybridizes with SEQ ID NO:1, or a functional derivative of the isolated DNA molecule which hybridizes with SEQ ID NO:1. In one embodiment of this aspect of the invention, the isolated DNA molecule has the nucleotide sequence of SEQ ID NO:1, i.e., 100% complementarity (sequence identity) to SEQ ID NO:1.

The present invention also is directed to an isolated DNA molecule encoding apoptosis-induced eIF-5A, wherein the DNA molecule hybridizes with SEQ ID NO:3, SEQ ID NO:4 or SEQ ID NO:5, or functional derivatives of the isolated DNA molecule which hybridize with SEQ ID NO:3, SEQ ID NO:4 or SEQ ID NO:5.

The present invention also is directed to an isolated DNA molecule encoding the 3' end of apoptosis-induced DHS, wherein the DNA molecule hybridizes with SEQ ID NO:6, or a functional derivative of the isolated DNA molecule which hybridizes with SEQ ID NO:6. In one embodiment of this aspect of the invention, the isolated DNA

12

molecule has the nucleotide sequence of SEQ ID NO:6, i.e., 100% complementarity (sequence identity) to SEQ ID NO:6.

The present invention also is directed to an isolated DNA molecule encoding the 3' end of apoptosis-induced DHS, wherein the DNA molecule hybridizes with SEQ ID NO:8, or functional derivatives of the isolated DNA molecule which hybridize with SEQ ID NO:8.

In another embodiment of the invention, there is provided an isolated protein encoded by a DNA molecule as described herein above, or a functional derivative thereof. A preferred protein has the amino acid sequence of SEQ ID NO:2, or is a functional derivative thereof. Another preferred protein has the amino acid sequence of SEQ ID NO: 7, or is a functional derivative thereof.

Also provided herein is an oligonucleotide or polynucleotide encoding an antisense RNA molecule which is complementary to a corresponding portion of an RNA transcript of a DNA molecule described herein above, wherein the oligonucleotide or polynucleotide hybridizes with the RNA transcript such that expression of endogenous apoptosis-induced eIF-5A is altered in a cell, tissue or organism. In another embodiment of this aspect of the invention, the antisense oligonucleotide or polynucleotide is an RNA molecule that hybridizes to a corresponding portion of an RNA transcript of a DNA molecule described herein above, such that expression of endogenous apoptosis-induced eIF-5A is altered in a cell, tissue or organism. The antisense oligonucleotide or polynucleotide can be full length or preferably has about six to about 100 nucleotides, more preferably about 8 to about 50 nucleotides, even more preferably about 8 to about 30 nucleotides.

The antisense oligonucleotide or polynucleotide may be substantially complementary to a corresponding portion of one strand of a DNA molecule encoding apoptosis-induced eIF-5A, wherein the DNA molecule encoding apoptosis-induced eIF-5A hybridizes with SEQ ID NO:1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:5 or with a combination thereof, or is substantially complementary to at least a corresponding portion of an RNA sequence encoded by the DNA molecule encoding apoptosis-induced eIF-5A. In one embodiment of the invention, the antisense oligonucleotide or polynucleotide is substantially complementary to a corresponding portion of one strand of the nucleotide sequence SEQ ID NO:1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:5 or with a combination thereof, or the RNA transcript transcribed from SEQ ID NO:1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:5 or with a combination thereof. In another embodiment, the antisense oligonucleotide is substantially complementary to a corresponding portion of the 5' non-coding portion or 3' non-coding portion of one strand of a DNA molecule encoding apoptosis-induced eIF-5A, wherein the DNA molecule hybridizes with SEQ ID NO:1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:5 or with a combination thereof.

Alternatively, the antisense oligonucleotide or polynucleotide may be substantially complementary to a corresponding portion of one strand of a DNA molecule encoding apoptosis-induced DHS, wherein the DNA molecule encoding apoptosis-induced DHS hybridizes with SEQ ID NO:6, SEQ ID NO: 8, or any combination thereof, or is substantially complementary to at least a corresponding portion of an RNA sequence transcribed from SEQ ID NO:6, SEQ ID NO: 8. In one embodiment of the invention, the antisense oligonucleotide or polynucleotide is substantially complementary to a

14

corresponding portion of one strand of the nucleotide sequence SEQ ID NO:6, SEQ ID NO: 8 or a combination thereof, or the RNA transcript encoded is substantially complementary to a corresponding portion of an RNA sequence encoded by a DNA molecule encoding apoptosis-induced DHS. In another embodiment, the antisense oligonucleotide is substantially complementary to a corresponding portion of the 5' non-coding region or 3' non-coding region of one strand of a DNA molecule encoding apoptosis-induced DHS, wherein the DNA molecule hybridizes with SEQ ID NO:6, SEQ ID NO: 8 or a combination thereof.

In another embodiment of the invention, a vector for transient expression in animal/ human cells, comprising (a) an antisense oligo- or polynucleotide substantially complementary to (1) a corresponding portion of one strand of a DNA molecule encoding apoptosis-induced eIF-5A, wherein the DNA molecule encoding apoptosis-induced eIF-5A hybridizes with SEQ ID NO:1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:5, or (2) a corresponding portion of an RNA sequence encoded by the DNA molecule encoding apoptosis-induced eIF-5A; and (b) regulatory sequences operatively linked to the antisense oligo- or polynucleotide such that the antisense oligo- or polynucleotide is transiently expressed in animal/human cells is provided.

In another embodiment of the invention, a vector for transient expression in animal/human cells, comprising (a) an antisense oligo- or polynucleotide substantially complementary to (1) a corresponding portion of one strand of a DNA molecule encoding apoptosis-induced DHS, wherein the DNA molecule encoding apoptosis-induced DHS hybridizes with SEQ ID NO:6, SEQ ID NO: 8 or (2) a corresponding portion of an RNA sequence encoded by the DNA molecule encoding apoptosis-induced DHS; and (b)

15

regulatory sequences operatively linked to the antisense oligo- or polynucleotide such that the antisense oligo- or polynucleotide is transiently expressed in animal/human cells is provided.

The invention also provides an animal/human cell, tissue or organ expressing a vector or combination of vectors as described above.

In yet another embodiment, the present invention provides a method of producing an animal/ human cell, tissue, or organ having a reduced level of apoptosis-induced DHS, apoptosis-induced eIF-5A, or both compared to an unmodified animal/human cell, tissue or organ, comprising: (1) introducing into the animal/human cell, tissue or organ a vector or combination of vectors as described above; (2) allowing transient expression of said vector or combination of vectors; (3) assaying said animal/human cell, tissue or organ for reduced apoptosis-induced DHS activity and/or eIF-5A activity and/or amelioration of the symptoms of apoptosis-induced disease.

Animals/humans or animal/human cells, tissues or organs treated or altered according to the methods of the present invention preferably exhibit reduced apoptosis-induced DHS expression, reduced apoptosis-induced eIF-5A activity, or both, and reduction or elimination of apoptosis-induced disease states.

In another embodiment, the present invention provides a method of modulating expression of endogenous apoptosis-induced eIF-5A in animal/human cells, tissues and organs, said method comprising: (1) introducing into animal/human cells, tissues or organs a vector comprising (a) an antisense oligo- or polynucleotide complementary to (i) at least a portion of one strand of a DNA molecule encoding endogenous apoptosis-induced eIF-5A, wherein the DNA molecule encoding the endogenous apoptosis-induced

eIF-5A hybridizes with SEQ ID NO:1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:5, or (ii) at least a portion of an RNA sequence encoded by the endogenous apoptosis-induced eIF-5A gene; and (b) regulatory sequences operatively linked to the antisense oligo- or polynucleotide such that the antisense oligo- or polynucleotide is expressed; and (2) transiently transcribing said antisense oligo- or polynucleotide whereby said transcript binds to said endogenous RNA and expression of said apoptosis-induced eIF-5A gene is modulated, preferably reduced.

In yet another embodiment, the present invention provides a method of modulating, preferably reducing, expression of endogenous apoptosis-induced DHS in animal/human cells, tissues and organs, said method comprising: (1) introducing into animal/human cells, tissues or organs a vector comprising (a) an antisense oligo- or polynucleotide complementary to (i) at least a portion of one strand of a DNA molecule encoding endogenous apoptosis-induced DHS, wherein the DNA molecule encoding the endogenous apoptosis-induced DHS hybridizes with SEQ ID NO:6, SEQ ID NO: 8, or (ii) at least a portion of an RNA sequence encoded by the endogenous apoptosis-induced DHS gene; and (b) regulatory sequences operatively linked to the antisense oligo- or polynucleotide such that the antisense oligo- or polynucleotide is expressed; and (2) transiently transcribing said antisense oligo- or polynucleotide whereby said transcript binds to said endogenous RNA and expression of said apoptosis-induced DHS gene is modulated, preferably reduced.

BRIEF DESCRIPTION OF THE DRAWINGS

17

Fig. 1 depicts a DNA laddering experiment in which the degree of apoptosis in dispersed cells of superovulated rat corpora lutea was examined. Superovulation was induced by subcutaneous injection with 500 µg of prostaglandin $F_{2\alpha}$. Control rats were treated with an equivalent volume of saline solution. Fifteen to thirty minutes later, the ovaries were removed and minced with collagenase. The dispersed cells from rats treated with prostaglandin $F_{2\alpha}$ were incubated in 10 mm glutamine + 10 mm spermidine for 1 hour and for a further 5 hours in 10 mm glutamine without spermidine (lane 2) or in 10 mm glutamine + 10 mm spermidine for 1 hour and for a further 5 hours in 10 mm glutamine + 1 mm spermidine (lane 3). Control cells from rats treated with saline were dispersed with collagenase and incubated for 1 hour and a futher 5 hours in glutamine only (lane 1). 500 ng of DNA from each sample was labeled with $[\alpha\text{-}^{32}P]$-dCTP using klenow enzyme, separated on a 1.8 % agarose gel, and exposed to film for 24 hours.

FIG. 2 depicts a DNA laddering experiment in which the degree of apoptosis in superovulated rat corpus lutea was examined in rats treated with spermidine prior to exposure to prostaglandin $F_{2\alpha}$. Superovulated rats were injected subcutaneously with 1 mg/100 g body weight of spermidine, delivered in three equal doses of 0.333 mg/100 g body weight, 24, 12, and 2 hours prior to a subcutaneous injection with 500 µg prostaglandin $F_{2\alpha}$. Control rats were divided into three sets, those which received no injections, those which received three injections of spermidine but no prostaglandin $F_{2\alpha}$, and those which received three injections with an equivalent volume of saline prior to prostaglandin F-,,x treatment. Ovaries were removed front the rats either 1 hour and 35 minutes or 3 hours and 45 minutes after prostaglandin treatment and used for the isolation

of DNA. 500 ng of DNA from each sample was labeled with $[\alpha\text{-}^{32}P]$-dCTP using Klenow

enzyme, separated on a 1.8 % agarose gel, and exposed to film for 24 hours. Lane 1, no

injections (animals were sacrificed at the same time as for lanes 3-5); lane 2, three

injections with spermidine (animals were sacrificed at the same time as for lanes 3-5);

lane 3, three injections with saline followed by injection with prostaglandin $F_{2\alpha}$ (animals

were sacrificed 1 h and 35 min after treatment with prostaglandin $F_{2\alpha}$); lane 4, three

injections with spermidine followed by injection with prostaglandin $F_{2\alpha}$ (animals were

sacrificed 1 h and 35 min after treatment with prostaglandin $F_{2\alpha}$); lane 5, three injections

with spermidine followed by injection with prostaglandin $F_{2\alpha}$ (animals were sacrificed 1

h and 35 min after treatment with prostaglandin $F_{2\alpha}$); lane 6, three injections with

spermidine followed by injection with prostaglandin $F_{2\alpha}$ (animals were sacrificed 3 h and

45 min after treatment with prostaglandin $F_{2\alpha}$); lane 7, three injections with spermidine

followed by injection with prostaglandin $F_{2\alpha}$ (animals were sacrificed 3 h and 45 min

after treatment with prostaglandin $F_{2\alpha}$).

FIG. 3 depicts the nucleotide sequence and derived amino acid sequence of the 3'

end of rat apoptosis-induced eIF-5A cDNA obtained by RT-PCR using as a template total

RNA isolated from apoptosing rat corpus luteum 24 hours after the induction of

apoptosis by treatment with prostaglandin $F_{2\alpha}$. This partial – length sequence was used

for Northern blot analyses. The cDNA sequence is SEQ ID NO. 11. The amino acid

sequence is SEQ ID NO. 12.

FIG. 4 depicts the nucleotide sequence and derived amino acid sequence of the 5'

end of rat apoptosis-induced eIF-5A cDNA obtained by RT-PCR using as a template total

RNA isolated from rat corpus luteum 24 hours after the induction of apoptosis by

treatment with prostaglandin $F_{2\alpha}$. The cDNA sequence is SEQ ID NO. 15. The amino

acid sequence is SEQ ID NO. 16.

FIG. 5 depicts the nucleotide sequence of rat corpus luteum apoptosis-induced

eIF-5A full-length cDNA and the derived amino acid sequence obtained by RT-PCR of

total RNA isolated from apoptosing rat corpus luteum. The cDNA sequence is SEQ ID

NO. 1. The amino acid sequence is SEQ ID NO. 2.

FIG. 6 is an alignment of the full-length nucleotide sequence of rat corpus luteum

apoptosis-induced eIF-5A cDNA with the nucleotide sequence of human eIF-5A

(Accession number BC000751or NM_001970). Human nucleotide sequence (Accession

number BC000751or NM_001970) is SEQ ID NO. 3.

FIG. 7 is an alignment of the full-length nucleotide sequence of rat corpus luteum

apoptosis-induced eIF-5A cDNA with the nucleotide sequence of human eIF-5A

(Accession number NM-020390). Human nucleotide sequence (Accession number NM-

020390) is SEQ ID NO. 4.

FIG. 8 is an alignment of the full-length nucleotide sequence of rat corpus luteum

apoptosis-induced eIF-5A cDNA with the nucleotide sequence of mouse eIF-5A

(Accession number BC003889). Murine nucleotide sequence (Accession number BC003889) is SEQ ID NO. 5.

FIG. 9 is an alignment of the derived full-length amino acid sequence of rat corpus luteum apoptosis-induced eIF-5A with the derived amino acid sequence of human eIF-5A (Accession number BC000751 or NM_001970).

FIG. 10 is an alignment of the derived full-length amino acid sequence of rat corpus luteum apoptosis-induced eIF-5A with the derived amino acid sequence of human eIF-5A (Accession number NM_020390).

FIG. 11 is an alignment of the derived full-length amino acid sequence of rat corpus luteum apoptosis-induced eIF-5A with the derived amino acid sequence of mouse eIF-5A (Accession number BC003889).

FIG. 12 is a restriction map of rat corpus luteum apoptosis-induced eIF-5A cDNA

FIG. 13 is a Southern blot of rat genomic DNA probed with ^{32}P-dCTP-labeled full-length rat corpus luteum apoptosis-induced eIF-5A cDNA. Genomic DNA was cut with restriction enzymes as indicated. Several isoforms of eIF-5A are detectable. The restriction fragment corresponding to the apoptosis-induced isoform of eIF-5A is indicated by a single arrow in the lanes labeled EcoR1 and BamH1, restriction enzymes for which there are no cut sites within the open reading frame. The two restriction

21

fragments of the apoptosis-induced isoform of eIF-5A obtained when genomic DNA is

cut with EcoRV, a restriction enzyme for which there is a cut site within the open reading

frame, are indicated by double arrows. The data indicate that the apoptosis-induced eIF-

5A is a single copy gene.

FIG. 14 is a Northern blot of total RNA isolated from apoptosing rat corpus

luteum at 0 hours, 1 hour, 8 hours and 24 hours after the induction of apoptosis by

treatment with prostaglandin $F_{2\alpha}$. The top panel is an autoradiograph of the Northern blot

probed with the ^{32}P-dCTP-labeled 3'-end of rat corpus luteum apoptosis-induced eIF-5A

cDNA. The lower panel is the ethidium bromide stained gel of total RNA. Each lane

contains 20 μg RNA.

FIG. 15 depicts the nucleotide sequence and derived amino acid sequence of the

3' end of rat apoptosis-induced DHS cDNA obtained by RT-PCR using as a template

total RNA isolated from apoptosing rat corpus luteum 24 hours after the induction of

apoptosis by treatment with prostaglandin $F_{2\alpha}$. The cDNA sequence is SEQ ID NO. 6.

The amino acid sequence is SEQ ID NO. 7.

FIG. 16 is a restriction map of partial-length rat apoptosis-induced DHS cDNA.

FIG. 17 is a Northern blot of total RNA isolated from apoptosing rat corpus

luteum at 0 hours, 1 hour, 8 hours and 24 hours after the induction of apoptosis by

treatment with prostaglandin $F_{2\alpha}$. The top panel is an autoradiograph of the Northern blot

probed with the ^{32}P-dCTP-labeled 3'-end of rat corpus luteum apoptosis-induced DHS

cDNA. The lower panel is the ethidium bromide stained gel of total RNA. Each lane

contains 20 μg RNA.

FIG. 18 is an alignment of the partial-length nucleotide sequence of rat corpus

luteum apoptosis-induced DHS cDNA with the nucleotide sequence of human DHS

(Accession number BC000333). Human nucleotide sequence (Accession number

BC000333) is SEQ ID NO. 8.

FIG. 19 depicts a DNA laddering experiment in which the degree of apoptosis in

superovulated rat corpus lutea was examined either 0, 1, or 24 hours after injection with

PGF-$_{2\alpha}$. In the 0 hour control, the ovaries were removed without PGF-$_{2\alpha}$ injection. DNA

was extracted from isolated ovaries at the indicated time points and 5 μg of DNA from

each sample was labeled with [α-^{32}P]-dCTP using Klenow enzyme. The labeled DNA

was separated on a 1.8 % agarose gel and the gel was dried and exposed to film for 24

hours. DNA laddering and increased end labeling of genomic DNA are evident within

one hour of PGF$_{2\alpha}$ treatment and to a greater degree by 24 hours after PGF$_{2\alpha}$ treatment.

FIG. 20 is an agarose gel of genomic DNA isolated from apoptosing rat corpus

luteum showing DNA laddering. Apoptosis was induced by treatment of rats with

prostaglandin F$_{2\alpha}$. Corresponding control animals were treated with saline instead of

prostaglandin F$_{2\alpha}$. Fifteen minutes after treatment with saline or prostaglandin F$_{2\alpha}$,

corpora lutea were removed from the animals. Genomic DNA was isolated from the

corpora lutea at 3 hours and 6 hours after removal of the tissue from the animals, labeled

with ^{32}P-dCTP, fractionated on an agarose gel and visualized by autoradiography. DNA laddering and increased end labeling of genomic DNA are evident 6 hours after removal of the tissue from the prostaglandin $F_{2\alpha}$-treated animals, but not at 3 hours after removal of the tissue.

FIG. 21 is a Southern blot of rat genomic DNA probed with ^{32}P-dCTP-labeled partial-length rat corpus luteum apoptosis-induced DHS cDNA. The genomic DNA was cut with EcoRV, a restriction enzyme which does not cut the partial-length cDNA used as a probe. Two restriction fragments are evident indicating that there are two copies of the gene or that the gene contains an intron with an EcoRV site.

DETAILED DESCRIPTION OF THE INVENTION

Methods and compositions are provided for altering the expression of apoptosis-induced DHS gene(s), apoptosis-induced eIF-5A gene(s) or both in animal cells. Reduction of expression of apoptosis-induced DHS and apoptosis-induced eIF-5A, either alone or in combination, in animals results in reduced apoptosis and gives rise to novel methods and compositions for the treatment and prevention of diseases caused by, causing, or otherwise having an etiology associated with inappropriate, excessive, or premature apoptosis. As non-limiting examples, the methods and compositions of the present invention may be used to prevent or treat the following apoptosis-associated diseases and disorders: neurological/ neurodegerative disorders (e.g., Alzheimer's, Parkinson's, Huntington's, Amyotrophic Lateral Sclerosis (Lou Gehrig's Disease), autoimmune disorders (e.g., rheumatoid arthritis, systemic lupus erythematosus (SLE), multiple sclerosis), Duchenne Muscular Dystrophy (DMD), motor neuron disorders,

24

ischemia, chronic heart failure, stroke, infantile spinal muscular atrophy, cardiac arrest, renal failure, atopic dermatitis, sepsis and septic shock, AIDS, hepatitis, glaucoma, diabetes (type 1 and type 2), asthma, retinitis pigmentosa, osteoporosis, xenograft rejection, and burn injury.

Animals, preferably mammals, and more preferably humans may be treated using the compositions and methods of the present invention.

Methods and compositions are provided for modulating the expression of apoptosis-induced DHS gene(s), apoptosis-induced eIF-5A gene(s), or both in animal/human cells, tissues, and organs. Modulating expression of apoptosis-induced DHS and apoptosis-induced eIF-5A, either alone or in combination, in animal/human cells, tissues, or organs results in reduction or elimination of the symptoms of diseases attributable to inappropriate, premature, or excess apoptosis.

The general approach to modulating apoptosis, according to the present invention, will be to reduce the amount of active apoptosis-induced eIF-5A in a cell, thereby modulating the apoptotic pathways triggered by active apoptosis-induced eIF-5A.

The incidence of apoptosis may be inhibited, substantially decreased, or delayed by preventing transcription of the gene encoding apoptosis-induced eIF-5A, translation of apoptosis-induced eIF-5A RNA, and/ or activation of apoptosis-induced eIF-5A. Activation of apoptosis-induced eIF-5A may be inhibited, substantially decreased, or delayed by preventing transcription of the gene encoding apoptosis-induced DHS and/or translation of apoptosis-induced DHS mRNA. Activation of apoptosis-induced eIF-5A may also be inhibited, substantially decreased, or delayed by blocking the action of apoptosis-induced DHS on apoptosis-induced eIF-5A.

In certain embodiments, the present invention provides methods and compositions for modulating or altering apoptosis, and thus controlling diseases associated with the induction of apoptosis, by gene therapy.

In certain preferred embodiments, the present invention employs antisense constructs, particularly oligonucleotides, for use in modulating the function of nucleic acid molecules encoding apoptosis-induced DHS and/ or apoptosis-induced eIF-5A, ultimately modulating the amount of apoptosis-induced DHS and/ or apoptosis-induced eIF-5A produced and/ or the amount of activated apoptosis-induced eIF-5A produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding apoptosis-induced DHS and/ or apoptosis-induced eIF-5A.

Thus, in one embodiment of the invention, apoptosis-induced eIF-5A nucleotide sequences of the invention, fragments thereof, or combinations of such fragments, are introduced into animal/human cells or tissue in reverse orientation to modulate expression of the endogenous apoptosis-induced eIF-5A gene, thereby reducing the level of endogenous apoptosis-induced eIF-5A protein, and thereby reducing and/or preventing expression of the genes that mediate apoptosis.

In another embodiment of the invention, apoptosis-induced DHS nucleotide sequence of the invention, fragments thereof, or combinations of such fragments, are introduced into animal/human cells or tissue in reverse orientation to reduce expression of the endogenous apoptosis-induced DHS gene, and thereby reduce the level of endogenous apoptosis-induced DHS protein, and thereby reducing and/or preventing

26

activation of apoptosis-induced eIF-5a and the ensuing expression of the genes that mediate apoptosis.

In yet another embodiment of the present invention, both the apoptosis-induced DHS sequence and the apoptosis-induced eIF-5A sequence can be used together to reduce the levels of endogenous apoptosis-induced DHS and apoptosis-induced eIF-5A proteins.

Apoptosis in cells, tissues, organs, and organisms can also be controlled through the use of other gene therapy methods, such as cosuppression of apoptosis-induced eIF-5A and/ or apoptosis-induced DHS. Additional methods include the use of homologous recombination to mutate endogenous apoptosis-induced eIF-5A and/ or apoptosis-induced DHS, such that fewer copies of functional, active apoptosis-induced eIF-5A and/ or apoptosis-induced DHS proteins are produced.

Alternatively, the activation of apoptosis-induced eIF-5A by apoptosis-induced DHS can be reduced or blocked by administering chemical inhibitors of the DHS enzymatic reaction. For example, the onset of DNA laddering reflecting apoptosis is delayed in rat corpus luteum when the animals are treated with spermidine, an inhibitor of the DHS reaction after induction of apoptosis by injection of prostaglandin $F_{2\alpha}$ (FIGS. 1 and 2). Jakus, J., Wolff, E. C., Park, M. H., Folk, J. E., "Features of the spermidine binding site of deoxyhypusine synthase as derived from inhibition studies," *Journal of Biological Chemistry* 268: 13151-13159 (1993).

Apoptosis also may be inhibited, substantially decreased, or delayed by adding agents that degrade apoptosis-induced eIF-5A DNA, RNA, or protein, or that degrade apoptosis-induced DHS DNA, RNA, or protein, thereby preventing the activation of

apoptosis-induced eIF-5A by apoptosis-induced DHS. In another embodiment of the invention, inhibition of expression of endogenous mammalian apoptosis-induced DHS, apoptosis-induced eIF-5A, or both is affected through the use of ribozymes.

Genetically modified animal/human cells, tissues or organs are defined herein as animal/human cells, tissues or organs containing incorporated heterologous or homologous apoptosis-induced DHS DNA or modified DNA or some portion of heterologous apoptosis-induced DHS DNA or homologous apoptosis-induced DHS DNA that is being transiently expressed. Alternatively, genetically modified animal/human cells, tissues or organs may contain incorporated heterologous or homologous apoptosis-induced eIF-5A DNA or modified DNA or some portion of heterologous apoptosis-induced eIF-5A DNA or homologous apoptosis-induced eIF-5A DNA that is being transiently expressed. The altered genetic material may encode a protein, comprise a regulatory or control sequence, or may be or include an antisense sequence or encode an antisense RNA which is antisense to the endogenous apoptosis-induced DHS or eIF-5A DNA or mRNA sequence or portion thereof of the animal/human cells, tissue or organ.

The term "hybridization" as used herein is generally used to mean hybridization of nucleic acids at appropriate conditions of stringency as would be readily evident to those skilled in the art depending upon the nature of the probe sequence and target sequences. Conditions of hybridization and washing are well known in the art, and the adjustment of conditions depending upon the desired stringency by varying incubation time, temperature and/or ionic strength of the solution are readily accomplished. See, e.g., Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Press, Cold Spring Harbor, New York, 1989.

The choice of conditions is dictated by the length of the sequences being hybridized, in particular, the length of the probe sequence, the relative G-C content of the nucleic acids and the amount of mismatches to be permitted. Low stringency conditions are preferred when partial hybridization between strands that have lesser degrees of complementarity is desired. When perfect or near perfect complementarity is desired, high stringency conditions are preferred. For typical high stringency conditions, the hybridization solution contains 6X S.S.C., 0.01 M EDTA, 1X Denhardt's solution and 0.5% SDS. Hybridization is carried out at about 68°C for about 3 to 4 hours for fragments of cloned DNA and for about 12 to about 16 hours for total eukaryotic DNA. For lower stringencies, the temperature of hybridization is reduced to about 42°C below the melting temperature (T_m) of the duplex. The T_m is known to be a function of the G-C content and duplex length as well as the ionic strength of the solution.

As used herein, the term "substantial sequence identity" or "substantial homology" is used to indicate that a nucleotide sequence or an amino acid sequence exhibits substantial structural or functional equivalence with another nucleotide or amino acid sequence. Any structural or functional differences between sequences having substantial sequence identity or substantial homology will be *de minimis*; that is, they will not affect the ability of the sequence to function as indicated in the desired application. Differences may be due to inherent variations in codon usage among different species, for example. Structural differences are considered *de minimis* if there is a significant amount of sequence overlap or similarity between two or more different sequences or if the different sequences exhibit similar physical characteristics even if the sequences differ in length or structure. Such characteristics include, for example, ability to hybridize under

29

defined conditions, or in the case of proteins, immunological crossreactivity, similar enzymatic activity, etc. Each of these characteristics can readily be determined by the skilled practitioner by art known methods.

Additionally, two nucleotide sequences are "substantially complementary" if the sequences have at least about 70 percent or greater, more preferably 80 percent or greater, even more preferably about 90 percent or greater, and most preferably about 95 percent or greater sequence similarity between them. Two amino acid sequences are substantially homologous if they have at least 50%, preferably at least 70%, more preferably at least 80%, even more preferably at least 90%, and most preferably at least 95% similarity between the active, or functionally relevant, portions of the polypeptides.

As used herein, the phrase "hybridizes to a corresponding portion" of a DNA or RNA molecule means that the molecule that hybridizes, e.g., oligonucleotide, polynucleotide, or an ~nucleotide sequence (in sense or antisense orientation) recognizes and hybridizes to a sequence in another nucleic acid molecule that is of approximately the same size and has enough sequence similarity thereto to effect hybridization under appropriate conditions. For example, a 100 nucleotide long antisense molecule from the 3' coding or non-coding region of rat DHS will recognize and hybridize to an approximately 100 nucleotide portion of a nucleotide sequence within the 3' coding or non-coding region, respectively of human DHS gene or any other animal DHS gene so long as there is about 70% or more sequence similarity between the two sequences. It is to be understood that the size of the "corresponding portion" will allow for some mismatches in hybridization such that the "corresponding portion" may be smaller or

30

larger than the molecule which hybridizes to it, for example 20-30% larger or smaller, preferably no more than about 12-15% larger or smaller.

The term "functional derivative" of a nucleic acid (or poly- or oligonucleotide) is used herein to mean a fragment, variant, homolog, or analog of the gene or nucleotide sequence encoding apoptosis-induced DHS or apoptosis-induced eIF-5A. A functional derivative may retain at least a portion of the function of the apoptosis-induced DHS or eIF-5A encoding DNA which permits its utility in accordance with the invention. Such function may include the ability to hybridize under low stringency conditions with native human apoptosis-induced DHS or eIF-5A or substantially homologous DNA from another animal which encodes apoptosis-induced DHS or eIF-5A or with an mRNA transcript thereof, or, in antisense orientation, to inhibit the transcription and/or translation of animal/human apoptosis-induced DHS or eIF-5A mRNA, or the like.

A "fragment" of the gene or DNA sequence refers to any subset of the molecule, e.g., a shorter polynucleotide or oligonucleotide. A "variant" refers to a molecule substantially similar to either the entire gene or a fragment thereof, such as a nucleotide substitution variant having one or more substituted nucleotides, but which maintains the ability to hybridize with the particular gene or to encode mRNA transcript which hybridizes with the native DNA. A "homolog" refers to a fragment or variant sequence from a different animal genus or species. An "analog" refers to a non-natural molecule substantially similar to or functioning in relation to either the entire molecule, a variant or a fragment thereof.

In the context of the present invention, "modulation" or "alteration" means a decrease or reduction (inhibition) in the expression of a gene. In the context of the

present invention, inhibition (reduction) is the preferred form of modulation of gene expression and mRNA is a preferred target. By "altered expression" or "modified expression" of a gene, e.g., the apoptosis-induced DHS gene or apoptosis-induced eIF-5A gene, is meant any process or result whereby the normal expression of the gene, for example, that expression occurring in an unmodified animal/human cell, tissue or organ, is changed in some way. As intended herein, modification or alteration in gene expression preferably is complete or partial reduction in the expression of the apoptosis-induced DHS gene or apoptosis-induced eIF-5A gene or both, but may also include a change in the timing of expression, or another state wherein the expression of the apoptosis-induced DHS gene or apoptosis-induced eIF-5A gene or both differs from that which would be most likely to occur naturally in an unmodified animal/human cell, tissue or organ. A preferred modulation or alteration is one which results in reduction of apoptosis-induced DHS production, apoptosis-induced eIF-5A production, or both by the animal/ human cell, tissue or organ compared to production in an unmodified animal/human cell, tissue or organ.

The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect

of such interference with target nucleic acid function is modulation of the expression of DHS and/ or eIF-5A and/ or the amount of activated eIF-5A produced.

In the particular context of antisense, as understood in the present invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding

is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed.

As used herein, the term "target cells" refers to cells undergoing or predicted to undergo excessive, premature, and/ or inappropriate apoptosis.

As used herein, the terms "target nucleic acid" and "target oligonuceotide" describe an oligonucleotide, preferably within a cell, to which an antisense oligonucleotide of the present invention hybridizes. As used herein, the terms "target nucleic acid" and "target oligonuceotide" also describe an oligonucleotide, preferably within a cell, the expression of which is suppressed by cosuppression techniques, as described herein. Further as used herein, the terms "target nucleic acid" and "target oligonuceotide" also describe an oligonucleotide, preferably within a cell, which are mutated by homologous recombination techniques described herein. Target nucleic acids and target oligonuceotides preferably comprise genomic DNA, pre-RNA, cDNA, or mRNA.

As used herein, the terms, "nucleic acid encoding apoptosis-induced DHS", and "nucleic acid encoding apoptosis-induced eIF-5A" encompass DNA encoding apoptosis-induced DHS, DNA encoding apoptosis-induced eIF-5A, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA.

A full length cDNA sequence encoding a rat eIF-5A gene exhibiting apoptosis-induced expression has been isolated by reverse transcriptase mediated polymerase chain reaction (RT -PCR) using RNA isolated from apoptosing corpus luteum as a template. Polynucleotide probes corresponding to selected regions of the isolated rat corpus luteum

34

cDNA sequence as well as the full length rat corpus luteum cDNA were used to determine the presence of mRNA encoding eIF-5A in apoptosing rat corpus luteum.

The apoptosis-induced eIF-5A gene of the present invention was isolated by using RT-PCR. The 3' end of the gene was isolated first using primers designed according to the yeast, fungi, and human eIF-5A cDNA sequences. The upstream primer used to isolate the 3'end of the rat eIF-5A gene is a 20 nucleotide degenerate primer: 5'TCSAARACHGGNAAGCAYGG 3' (SEQ ID NO. 9), wherein S is selected from C and G; R is selected from A and G; H is selected from A, T, and C; Y is selected from C and T; and N is any nucleic acid.

The downstream primer used to isolate the 3'end of the rat eIF-5A gene contains 42 nucleotides:
5' GCGAAGCTTCCATGGCTCGAGTTTTTTTTTTTTTTTTTTTT 3' (SEQ ID NO.
                    XhoI
10). Using 5 μg of the downstream primer, a first strand of cDNA was isolated using standard RT-PCR. The first strand was then used as a template in a RT-PCR using both the upstream and downstream primers.

The PCR conditions were 94°C, 5 min, (94°C 1 min, 48°C 1min, 72°C 2 min for 45 cycles), and 72°C 15 min. Separation of the RT-PCR products on an agarose gel revealed the presence a 900 bp fragment, which was subcloned into pBluescript™ (Stratagene Cloning Systems, LaJolla, CA) using blunt end ligation and sequenced (SEQ ID NO. 11).

The 5' end of the rat apoptosis-induced eIF-5A gene was isolated using an upstream primer designed according to human eIF-5A cDNA sequences and a downstream primer designed according to the 3' end of the RT-PCR fragment. The upstream primer is a 24 nucleotide primer:

35

5' CAGG<u>TCTAGA</u>GTTGGAATCGAAGC 3'
        XbaI

(SEQ ID NO.13). The downstream primer contains 30 nucleotides:

5' ATAT<u>CTCGAG</u>CCTTGATTGCAACAGCTGCC 3'
        XhoI

(SEQ ID NO. 14).

Using 5 µg of the downstream primer, a first strand of cDNA was isolated using standard

RT-PCR. The first strand was then used as a template in a RT-PCR using both the

upstream and downstream primers.

    The PCR conditions were 94°C, 5 min, (94°C 1 min, 48°C 1min, 72°C 2 min for

45 cycles), and 72°C 15 min. Separation of the RT-PCR products on an agarose gel

revealed the presence a 500 bp fragment, which was subcloned into pBluescript™

(Stratagene Cloning Systems, LsJolla, CA) using XbaI and XhoI cloning sites present in

the upstream and downstream primers, respectively, and sequenced (SEQ ID NO. 15).

    The sequences of the 3' and 5' ends of the rat apoptosis-induced eIF-5A (SEQ ID

NO. 11 (see also, FIG. 3) and SEQ ID NO. 15 (see also, FIG. 4), respectively) overlapped

and gave rise to the full-length cDNA sequence (SEQ ID NO. 1 (see also, FIG. 5)). This

full-length sequence was aligned and compared with sequences in the GeneBank data

base. The results showed that the rat full-length cDNA sequence encodes eIF-5A. The

rat apoptosis-induced eIF-5A cDNA also aligned with human and mouse eIF-5A

sequences. See FIGS. 6 through 11.

    The nucleotide sequence of the rat apoptosis-induced cDNA clone is shown in

SEQ ID NO:1. The cDNA clone encodes a 154 amino acid polypeptide (SEQ ID NO: 2)

having a calculated molecular mass of 16.8 KDa.

Based on the expression pattern of the identified gene in developing and regressing rat corpus luteum, it is involved in apoptosis. Thus, the cDNA sequence of the invention encoding rat apoptosis-induced eIF-5A can be used as a probe to isolate apoptosis-induced genes from other animal/human cells, tissues and organs, using guidance provided herein and techniques well known to those skilled in the art.

The rat apoptosis-induced eIF-5A gene is a member of an eIF-5A gene family. Genomic Southern blot analysis of rat DNA was carried out using genomic DNA extracted from corpus luteum. The DNA was cut with various restriction enzymes that recognize a single site within the coding region of the eIF-5A gene or which do not recognize any sites within the open reading frame of the eIF-5A gene. A restriction map for rat apoptosis eIF-5A is shown in FIG. 12.

Restriction enzyme digested rat corpus luteum genomic DNA was probed with ^{32}P-dCTP-labeled full length eIF-5A cDNA. Hybridization under high stringency conditions revealed hybridization of the full length cDNA probe to several restriction fragments for each restriction enzyme digested DNA sample indicating the presence of several isoforms of eIF-5A. Of particular note, when rat genomic DNA was digested with EcoRV, which has a restriction site within the open reading frame of apoptosis-induced eIF-5A (FIG. 12), two restriction fragments of the apoptosis-induced isoform of eIF-5A were detectable in the Southern blot (FIG. 13). These results suggest that the apoptosis-induced eIF-5A is a single copy gene in rat. As shown in FIGS. 6 through 11, the eIF-5A gene is highly conserved across species, and so it would be expected that there is a significant amount of conservation between isoforms within any species.

Northern blots of apoptosing rat corpus luteum total RNA probed with full length rat apoptosis-induced eIF-5A cDNA show that the expression of apoptosis-induced eIF-5A is significantly enhanced after induction of apoptosis by treatment with prostaglandin $F_{2\alpha}$. Expression of eIF-5A was low at time zero, increased substantially within 1 hour of treatment, increased still more within 8 hours of treatment and decreased again slightly within 24 hours of treatment (FIG. 14).

Apoptosis-induced eIF-5A is a suitable target for regulation of apoptosis, including apoptosis underlying disease processes, since it likely acts in the post-transcriptional regulation of downstream effectors and transcription factors involved in the apoptotic pathway. Thus, it is expected that by substantially repressing the expression of the apoptosis-induced eIF-5A gene in animal/human cells, tissues or organs, diseases attributable to apoptosis can be controlled.

Alternatively, it is expected that by substantially enhancing the expression of the apoptosis-induced eIF-5A gene in animal/human cells, tissues or organs, diseases attributable to an inability to induce apoptosis (e.g cancer) can be controlled.

A partial-length cDNA corresponding to another gene, DHS, which is involved in the induction of apoptosis in animal/human cells, tissues, and organs, has also been isolated and sequenced herein and, like eIF-5A, it can be used to alter apoptosis and control apoptosis-related diseases. The partial-length apoptosis-induced DHS cDNA clone was isolated from apoptosing rat corpus luteum by RT-PCR using primers designed from the human DHS sequence. The upstream primer is a 20 nucleotide primer:

5' GTCTGTGTATTATTGGGCCC 3'

(SEQ ID NO. 17). The downstream primer contains 42 nucleotides:

38

5' GCGAAGCTTCCATGG<u>CTCGAG</u>TTTTTTTTTTTTTTTTTTTTT 3'
                 XhoI

(SEQ ID NO. 18). Using 5 μg of the downstream primer, a first strand of cDNA was isolated using standard RT-PCR. The first strand was then used as a template in a RT-PCR using both the upstream and downstream primers.

The PCR conditions were 94°C, 5 min, (94°C 1 min, 48°C 1min, 72°C 2 min for 45 cycles), and 72°C 15 min. Separation of the RT-PCR products on an agarose gel revealed the presence a 606 bp fragment, which was subcloned into pBluescript™ (Stratagene Cloning Systems, LaJolla, CA) using blunt end ligation and sequenced (SEQ ID NO.6).

The nucleotide and derived amino acid sequences for the 3' end of rat apoptosis-induced DHS (SEQ ID NO. 6 and SEQ ID NO. 7, respectively) are shown in FIG. 15. A restriction map of partial-length rat apoptosis-induced DHS cDNA is shown in FIG. 16.

As is the case with the eIF-5A gene sequence described herein, the DHS sequence of the present invention can be used to isolate apoptosis-induced DHS genes from other animals, including humans. Isolation of DHS sequences from animals and human can be achieved using art known methods and guidance provided herein, based on sequence similarities of at least 80% across species.

The eIF-5A and DHS genes of the invention are up-regulated during apoptosis. Northern blot analyses demonstrate that apoptosis-induced eIF-5A and DHS are both up-regulated in rat corpus luteum over a 24 hour period following induction of corpus luteum apoptosis by treatment with prostaglandin $F_{2\alpha}$ (FIGS. 14 and 17).

For the identification and isolation of the apoptosis-induced DHS gene and eIF-5A gene, in general, preparation of plasmid DNA, restriction enzyme digestion, agarose

gel electrophoresis of DNA, polyacrylamide gel electrophoresis of protein, PCR, RT -

PCR, Southern blots, Northern blots, DNA ligation and bacterial transformation were

carried out using conventional methods well-known in the art. See, e.g., Sambrook, J. et

al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Cold

Spring Harbor, NY, 1989. Techniques of nucleic acid hybridization are disclosed by

Sambrook.

In certain embodiments, methods and compositions are provided for modulating

apoptosis, and thus controlling diseases associated with apoptosis, by gene therapy. The

cloned apoptosis-induced eIF-5A gene, fragment(s) thereof, or cloned apoptosis-induced

DHS gene or fragment(s) thereof, or combinations of apoptosis-induced eIF-5A and

apoptosis-induced DHS sequences, when introduced in reverse orientation (antisense)

into animal/human cells, tissues, organs, or organisms and transiently expressed under the

control of a promoter can be used to reduce or prevent apoptosis in said cells, tissues,

organs, or organisms and, further, to control apoptosis related diseases in said cells,

tissues or organs. Selected antisense sequences from other animals and human which

share sufficient sequence identity with the rat apoptosis-induced DHS gene or rat

apoptosis-induced eIF-5A gene can be used to achieve similar genetic modification.

Using methods of the invention, antisense oligonucleotides corresponding to

regions of the apoptosis-induced eIF-5A nucleotide sequence, fragments thereof, or

combinations of such fragments, and/ or the apoptosis-induced DHS nucleotide sequence,

fragments thereof, or combinations of such fragments can be used to inhibit the

expression of apoptosis-induced eIF-5A protein and/ or apoptosis-induced DHS protein

in animal/human cells and tissues.

It is also possible to inhibit expression of apoptosis-induced eIF-5A by inactivating the gene coding for apoptosis-induced eIF-5A in a cell. Such inactivation can occur by deleting the gene in the cell or by introducing a deletion or mutation into the gene thereby inactivating the gene. The gene may also be inactivated by inserting into the gene another DNA fragment such that expression of the endogenous apoptosis-induced eIF-5A protein does not occur. Likewise, it is possible to inhibit activation of apoptosis-induced eIF-5A by inactivating the gene coding for apoptosis-induced DHS in a cell. Methods for introducing mutations, such as deletions and insertions, into genes in eukaryotic cells are known in the art. See, for example, U.S. Pat. No. 5,464,764, which is incorporated herein in its entirety. Oligonucleotides and expression vectors useful for mutation of genes in cells may be made according to methods known in the art and guidance provided herein; for example, methods useful for making and expressing antisense oligonucleotides may be used to make oligonucleotides and expression vectors useful for mutating genes in cells.

It is also possible to inhibit expression of apoptosis-induced eIF-5A by suppressing expression of the gene coding for apoptosis-induced eIF-5A in a cell. Such inactivation can be accomplished via cosuppression, e.g., by introducing nucleotide sequence(s) coding for apoptosis-induced eIF-5A into a cell such that cosuppression occurs. Likewise, it is possible to inhibit activation of apoptosis-induced eIF-5A by suppressing the expression of the gene coding for apoptosis-induced DHS in a cell via cosuppression. Oligonucleotides and expression vectors useful for cosuppression may be made according to methods known in the art and guidance provided herein; for example, methods useful for making and expressing antisense oligonucleotides may be used to

41

make oligonucleotides and expression vectors useful for cosuppression. Methods for cosuppression are known in the art. See, for example, U.S. Pat. No. 5,686,649, which is incorporated herein in its entirety.

One result of the genetic modification (through, e.g., antisense, mutation, or cosuppression) is a reduction in the amount of endogenous translatable apoptosis-induced DHS-encoding mRNA. Consequently, the amount of apoptosis-induced DHS protein produced is reduced, thereby reducing the amount of activated eIF-5A, which in turn reduces translation of apoptosis-induced proteins. Apoptosis is thus inhibited or delayed, since *de novo* protein synthesis is required for the onset of apoptosis.

Another result of the genetic modification is a reduction in the amount of endogenous translatable apoptosis-induced eIF-5A-encoding mRNA. Consequently, the amount of apoptosis-induced eIF-5A protein is reduced, which in turn reduces translation of apoptosis-induced proteins. Apoptosis is thus inhibited or delayed, since *de novo* protein synthesis is required for the onset of apoptosis.

Alternatively, the activation of apoptosis-induced eIF-5A by apoptosis-induced DHS can be reduced or blocked by administering chemical inhibitors of the DHS enzymatic reaction. For example, the onset of DNA laddering reflecting apoptosis is delayed in rat corpus luteum when the animals are treated with spermidine, an inhibitor of the DHS reaction after induction of apoptosis by injection of prostaglandin $F_{2\alpha}$ (FIGS. 1 and 2). Jakus, J., Wolff, E. C., Park, M. H., Folk, J. E., "Features of the spermidine binding site of deoxyhypusine synthase as derived from inhibition studies," *Journal of Biological Chemistry* 268: 13151-13159 (1993).

Apoptosis also may be inhibited or substantially decreased by adding agents that degrade apoptosis-induced eIF-5A DNA, RNA, or protein, or that degrade apoptosis-induced DHS DNA, RNA, or protein, thereby preventing the activation of apoptosis-induced eIF-5A by apoptosis-induced DHS. In another embodiment of the invention, inhibition of expression of endogenous mammalian apoptosis-induced DHS, apoptosis-induced eIF-5A, or both is affected through the use of ribozymes.

Thus, in certain embodiments, the present invention is directed to methods for modulating apoptosis in animal /human cells or tissues, and thus controlling diseases attributable to the induction of apoptosis, by drug therapy. Examples of suitable drugs include those that inhibit the activation of eIF-5A by DHS, those that inhibit the activation of eIF-5A by deoxyhypusine hydroxylase, those that inhibit transcription and/ or translation of apoptosis-induced DHS, those that inhibit transcription and/ or translation of apoptosis-induced deoxyhypusine hydroxylase, and those that inhibit transcription or translation of apoptosis-induced eIF-5A. Examples of drugs that inhibit the activation of eIF-5A by DHS are provided in Table 1. Toxicity and irritation data are also provided. Chemicals (drugs) that inhibit either the reaction catalyzed by deoxyhypusine synthase or the reaction catalyzed by deoxyhypusine hydroxylase (which reactions lead to the formation of apoptosis-induced eIF-5A protein) can be administered to animal/human tissues or organs by direct injection or using Alzet osmotic minipumps (e.g., DUROS). A minipump can be surgically implanted near the target tissue or organ and left in place for days to months. This implant can then be used to deliver small volumes of drug into the site immediately surrounding the target tissue, and the drug diffuses into the target tissue or organ along a concentration gradient. Information on

43

controlled drug delivery methods and devices is known in the art, and appropriate devices are commercially available. See, e.g., Marshall JW, et al., Stroke 32(1): 190-8 (2001); Urquhart J, et al., J. Intern. Med. 248(5): 357-76 (2000).

Table 1

Drug	Toxicity	Irritation
Spermidine	IPR-mouse LD50: 870 mg/kg	Irritant
1,3-Diamino-propane	ORL-rat LD50: 350 µg/kg IPR-mouse LD50: 296 mg/kg SKN-rabbit LD50: 200 µg/kg	SKN-rabbit 50 mg EYE-rabbit 1 mg
1,4-Diamino-butane (putrescine)	ORL-rat LD50: 463 mg/kg IPR-mouse LD50: 1750 mg/kg SKN-rabbit LD50: 1576 µg/kg	Data not available
1,7-Diamino-heptane	Data not available	Irritant
1,8-Diamino-octane	Data not available	SKN-rabbit 500 mg EYE-rabbit 100 mg

Drugs can also be administered to target cells, tissues, and organs of mammals using liposomes. Liposomes occluding the drug are administered intravenously. Targeting can be achieved by incorporating ligands to specific cell receptors into the liposomes. See, e.g., Kaneda, *Adv Drug Delivery Rev* 43: 197-205 (2000).

In one embodiment, the present invention is directed to an isolated DNA molecule encoding apoptosis-induced eIF-5A, wherein the DNA molecule hybridizes with SEQ ID NO:1, or a functional derivative of the isolated DNA molecule which hybridizes with SEQ ID NO:1. In one embodiment of this aspect of the invention, the isolated DNA molecule has the nucleotide sequence of SEQ ID NO:1, i.e., 100% complementarity (sequence identity) to SEQ ID NO:1.

The present invention also is directed to an isolated DNA molecule encoding apoptosis-induced eIF-5A, wherein the DNA molecule hybridizes with SEQ ID NO:3

(see also, FIG. 6, human sequence), SEQ ID NO:4 (see also, FIG. 7, human sequence), or

SEQ ID NO:5 (see also, FIG. 8, mouse sequence), or functional derivatives of the

isolated DNA molecule which hybridize with SEQ ID NO:3, SEQ ID NO:4 or SEQ ID

NO5.

The present invention also is directed to an isolated DNA molecule encoding the

3' end of apoptosis-induced DHS, wherein the DNA molecule hybridizes with SEQ ID

NO:6, or a functional derivative of the isolated DNA molecule which hybridizes with

SEQ ID NO:6. In one embodiment of this aspect of the invention, the isolated DNA

molecule has the nucleotide sequence of SEQ ID NO:6, i.e., 100% complementarity

(sequence identity) to SEQ ID NO:6.

The present invention also is directed to an isolated DNA molecule encoding the

3' end of apoptosis-induced DHS, wherein the DNA molecule hybridizes with SEQ ID

NO:8 (see also, FIG. 18, human sequence), or functional derivatives of the isolated DNA

molecule which hybridize with SEQ ID NO:8.

In another embodiment of the invention, there is provided an isolated protein

encoded by a DNA molecule as described herein above, or a functional derivative

thereof. A preferred protein has the amino acid sequence of SEQ ID NO:2, or is a

functional derivative thereof. Another preferred protein has the amino acid sequence of

SEQ ID NO: 7, or is a functional derivative thereof.

Also provided herein is an antisense oligonucleotide or polynucleotide encoding

an RNA molecule which is complementary to a corresponding portion of an RNA

transcript of a DNA molecule described herein above, wherein the oligonucleotide or

polynucleotide hybridizes with the RNA transcript such that expression of endogenous

45

apoptosis-induced eIF-5A is altered in cells, tissues, and/ or organisms. In another

embodiment of this aspect of the invention, the antisense oligonucleotide or

polynucleotide is an RNA molecule that hybridizes to a corresponding portion of an RNA

transcript of a DNA molecule described herein above, such that expression of

endogenous apoptosis-induced eIF-5A is altered in cells, tissues, and/ or organisms. The

antisense oligonucleotide or polynucleotide can be full length or preferably has about six

to about 100 nucleotides.

The antisense oligonucleotide or polynucleotide may be substantially

complementary to a corresponding portion of one strand of a DNA molecule encoding

apoptosis-induced eIF-5A, wherein the DNA molecule encoding apoptosis-induced eIF-

5A hybridizes with SEQ ID NO:1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:5 or with

a combination thereof, or is substantially complementary to at least a corresponding

portion of an RNA sequence encoded by the DNA molecule encoding apoptosis-induced

eIF-5A. In one embodiment of the invention, the antisense oligonucleotide or

polynucleotide is substantially complementary to a corresponding portion of one strand

of the nucleotide sequence SEQ ID NO:1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:5

or with a combination thereof, or the RNA transcript transcribed from SEQ ID NO:1,

SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:5 or with a combination thereof. In another

embodiment, the antisense oligonucleotide is substantially complementary to a

corresponding portion of the 5' non-coding portion or 3' non-coding portion of one strand

of a DNA molecule encoding apoptosis-induced eIF-5A, wherein the DNA molecule

hybridizes with SEQ ID NO:1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:5 or with a

combination thereof.

Alternatively, the antisense oligonucleotide or polynucleotide may be

substantially complementary to a corresponding portion of one strand of a DNA molecule

encoding apoptosis-induced DHS, wherein the DNA molecule encoding apoptosis-

induced DHS hybridizes with SEQ ID NO:6, SEQ ID NO: 8, or any combination thereof,

or is substantially complementary to at least a corresponding portion of an RNA sequence

transcribed from SEQ ID NO:6, SEQ ID NO: 8. In one embodiment of the invention, the

antisense oligonucleotide or polynucleotide is substantially complementary to a

corresponding portion of one strand of the nucleotide sequence SEQ ID NO:6, SEQ ID

NO: 8 or a combination thereof, or the RNA transcript encoded is substantially

complementary to a corresponding portion of an RNA sequence encoded by a DNA

molecule encoding apoptosis-induced DHS. In another embodiment, the antisense

oligonucleotide is substantially complementary to a corresponding portion of the 5' non-

coding region or 3' non-coding region of one strand of a DNA molecule encoding

apoptosis-induced DHS, wherein the DNA molecule hybridizes with SEQ ID NO:6, SEQ

ID NO: 8 or a combination thereof.

In another embodiment of the invention, a vector for transient expression in

animal/ human cells, comprising (a) an antisense oligo- or polynucleotide substantially

complementary to (1) a corresponding portion of one strand of a DNA molecule encoding

apoptosis-induced eIF-5A, wherein the DNA molecule encoding apoptosis-induced eIF-

5A hybridizes with SEQ ID NO:1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:5, or (2)

a corresponding portion of an RNA sequence encoded by the DNA molecule encoding

apoptosis-induced eIF-5A; and (b) regulatory sequences operatively linked to the

antisense oligo- or polynucleotide such that the antisense oligo- or polynucleotide is transiently expressed in animal/human cells is provided.

In another embodiment of the invention, a vector for transient expression in animal/human cells, comprising (a) an antisense oligo- or polynucleotide substantially complementary to (1) a corresponding portion of one strand of a DNA molecule encoding apoptosis-induced DHS, wherein the DNA molecule encoding apoptosis-induced DHS hybridizes with SEQ ID NO:6, SEQ ID NO: 8 or (2) a corresponding portion of an RNA sequence encoded by the DNA molecule encoding apoptosis-induced DHS; and (b) regulatory sequences operatively linked to the antisense oligo- or polynucleotide such that the antisense oligo- or polynucleotide is transiently expressed in animal/human cells is provided.

The regulatory sequences may include a promoter functional in the animal/human cell, which promoter may be inducible or constitutive. Optionally, the regulatory sequences include a polyadenylation signal.

The invention also provides an animal/human cell, tissue or organ expressing a vector or combination of vectors as described above.

In yet another embodiment, the present invention provides a method of producing an animal/ human cell, tissue, or organ having a reduced level of apoptosis-induced DHS, apoptosis-induced eIF-5A, or both compared to an unmodified animal/human cell, tissue or organ, comprising: (1) introducing into the animal/human cell, tissue or organ a vector or combination of vectors as described above; (2) allowing transient expression of said vector or combination of vectors; (3) assaying said animal/human cell, tissue or organ

48

for reduced apoptosis-induced DHS activity and/or eIF-5A activity and/or amelioration of the symptoms of apoptosis-induced disease.

Animals/humans or animal/human cells, tissues or organs treated or altered according to the methods of the present invention preferably exhibit reduced apoptosis-induced DHS expression, reduced apoptosis-induced eIF-5A activity, or both, and reduction or elimination of apoptosis-induced disease states.

In another embodiment, the present invention provides a method of inhibiting expression of endogenous apoptosis-induced eIF-5A in animal/human cells, tissues and organs, said method comprising: (1) introducing into animal/human cells, tissues or organs a vector comprising (a) an antisense oligo- or polynucleotide complementary to (i) at least a portion of one strand of a DNA molecule encoding endogenous apoptosis-induced eIF-5A, wherein the DNA molecule encoding the endogenous apoptosis-induced eIF-5A hybridizes with SEQ ID NO:1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:5, or (ii) at least a portion of an RNA sequence encoded by the endogenous apoptosis-induced eIF-5A gene; and (b) regulatory sequences operatively linked to the antisense oligo- or polynucleotide such that the antisense oligo- or polynucleotide is expressed; and (2) transiently transcribing said antisense oligo- or polynucleotide whereby said transcript binds to said endogenous RNA and expression of said apoptosis-induced eIF-5A gene is inhibited.

In yet another embodiment, the present invention provides a method of inhibiting expression of endogenous apoptosis-induced DHS in animal/human cells, tissues and organs, said method comprising: (1) introducing into animal/human cells, tissues or organs a vector comprising (a) an antisense oligo- or polynucleotide complementary to (i)

at least a portion of one strand of a DNA molecule encoding endogenous apoptosis-induced DHS, wherein the DNA molecule encoding the endogenous apoptosis-induced DHS hybridizes with SEQ ID NO:6, SEQ ID NO: 8, or (ii) at least a portion of an RNA sequence encoded by the endogenous apoptosis-induced DHS gene; and (b) regulatory sequences operatively linked to the antisense oligo- or polynucleotide such that the antisense oligo- or polynucleotide is expressed; and (2) transiently transcribing said antisense oligo- or polynucleotide whereby said transcript binds to said endogenous RNA and expression of said apoptosis-induced DHS gene is inhibited.

In order for a newly inserted gene or DNA sequence to be expressed, resulting in production of the protein which it encodes, or in the case of antisense DNA, to be transcribed, resulting in an antisense RNA molecule, the proper regulatory elements should be present in proper location and orientation with respect to the gene or DNA sequence. The regulatory regions may include a promoter, a 5'-non-translated leader sequence and a 3'-polyadenylation sequence as well as enhancers and other regulatory sequences.

Expression levels from a promoter which is useful for the present invention can be tested using conventional expression systems, for example by measuring levels of a reporter gene product, e.g., protein or mRNA in extracts of animal/human cells, tissues or organs into which the promoter/reporter gene have been introduced.

The present invention provides antisense oligonucleotides and polynucleotides complementary to the gene encoding rat apoptosis-induced eIF-5A or complementary to a gene or gene fragment from other animal/ human cells, tissues or organs, which hybridize with the rat apoptosis-induced eIF-5A gene under low to high stringency

conditions. The present invention also provides antisense oligonucleotides and polynucleotides complementary to the gene encoding rat apoptosis-induced DHS or complementary to a gene or gene fragment from other animal/human cells, tissues or organs, which hybridize with the rat apoptosis-induced DHS gene under low to high stringency conditions. Such antisense oligonucleotides should be at least about six nucleotides in length to provide minimal specificity of hybridization and may be complementary to one strand of DNA or mRNA encoding the apoptosis-induced gene or a portion thereof, or to flanking sequences in genomic DNA which are involved in regulating apoptosis-induced DHS or eIF-5A gene expression. The antisense oligonucleotide may be as large as 100 nucleotides or more and may extend in length up to and beyond the full coding sequence for which it is antisense. The antisense oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single stranded or double stranded.

The action of the antisense oligonucleotide may result in alteration, primarily inhibition, of apoptosis-induced DHS expression, apoptosis-induced eIF-5A expression or both in cells, tissues or organs. For a general discussion of antisense see: Alberts, et al., Molecular Biology of the Cell, 2nd ed., Garland Publishing, Inc. New York, New York,1989 (in particular pages 195-196, incorporated herein by reference).

Preferred antisense oligonucleotides are substantially complementary to a portion of the mRNA encoding apoptosis-induced DHS or apoptosis-induced eIF-5A, the portion of the mRNA being approximately the same size as the antisense oligonuleotide. For example, introduction of the full length cDNA clone encoding apoptosis-induced DHS or eIF-5A in an antisense orientation into an animal/human cell, tissue or organ is expected

to result in successfully altered apoptosis-induced DHS and/or eIF-5A gene expression. Introduction of partial sequences, targeted to specific portions of the apoptosis-induced DHS gene or apoptosis-induced eIF-5A gene or both, can be equally effective. The effectiveness of suppression of gene expression can be tested using art-known methods. For example, the effectiveness of suppression of gene expression can be tested *in vitro* by Northern blotting or western blotting; and the effectiveness of suppression of gene expression can be tested *in situ* by immunohistochemistry.

The minimal amount of homology required by the present invention is that sufficient to result in sufficient complementarity to provide recognition of the specific target RNA or DNA and inhibition or reduction of its translation or function while not affecting function of other RNA or DNA molecules and the expression of other genes. While the antisense oligonucleotides of the invention comprise sequences complementary to a corresponding portion of an RNA transcript of the apoptosis-induced DHS gene or apoptosis-induced eIF-5A gene, absolute complementarity, although preferred, is not required. The ability to hybridize may depend on the length of the antisense oligonucleotide and the degree of complementarity. Generally, the longer the hybridizing nucleic acid, the more base mismatches with the apoptosis-induced DHS target sequence or apoptosis-induced eIF-5a target sequence it may contain and still form a stable duplex. One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting temperature of the hybridized complex, for example.

The antisense RNA oligonucleotides may be generated intracellularly by transcription from exogenously introduced nucleic acid sequences. The antisense molecule may be delivered to a cell by transformation or transfection or infection with a

vector, such as a plasmid or virus into which is incorporated DNA encoding the antisense apoptosis-induced DHS sequence apoptosis-induced eIF-5A sequence or operably linked to appropriate regulatory elements, including a promoter. Within the cell the exogenous DNA sequence is expressed, producing an antisense RNA of the apoptosis-induced DHS gene or the apoptosis-induced eIF-5A gene.

Vectors can be plasmids, preferably, or may be viral or other vectors known in the art to replicate and express genes encoded thereon in animal/human cells or bacterial cells. The vector is transcribed to produce the desired antisense apoptosis-induced DHS RNA or apoptosis-induced eIF-5A RNA. Such plasmid or viral vectors can be constructed by recombinant DNA technology methods that are standard in the art. For example, the vector may be a plasmid vector containing a replication system functional in a prokaryotic host and an antisense oligonucleotide or polynucleotide according to the invention.

An oligonucleotide, preferably between about 6 and about 100 nucleotides in length and complementary to the target sequence of apoptosis-induced DHS or apoptosis-induced eIF-5A, may be prepared by recombinant nucleotide technologies or may be synthesized from mononucleotides or shorter oligonucleotides, for example. Automated synthesizers are applicable to chemical synthesis of the oligo-and polynucleotides of the invention. Procedures for constructing recombinant nucleotide molecules in accordance with the present invention are disclosed in Sambrook, et al., In: Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989), which is incorporated herein in its entirety. Oligonucleotides which encode antisense RNA complementary to apopotosis-induced DHS sequence can be prepared

using procedures well known to those in the art. Details concerning such procedures are provided in Maniatis, T. et al., Molecular mechanisms in the Control of Gene expression, eds., Nierlich, et al.,eds., Acad. Press, N.Y. (1976).

In an alternative embodiment of the invention, inhibition of expression of endogenous human/animal apoptosis-induced DHS, apoptosis-induced eIF-5A or both is the result of co-suppression through over-expression of an exogenous apoptosis-induced DHS or eIF-5A gene or gene fragment or both introduced into animal/human cells, tissues or organs. In this embodiment of the invention, a vector encoding apoptosis-induced DHS, apoptosis-induced eIF-5A or both in the sense orientation is introduced into the cells in the same manner as described herein for antisense molecules. Preferably, the apoptosis-induced DHS or apoptosis-induced eIF-5A is operatively linked to a strong promoter.

In another embodiment of the invention, inhibition of expression of endogenous mammalian apoptosis-induced DHS, apoptosis-induced eIF-5A or both is effected through the use of ribozymes. Ribozymes are RNA molecules exhibiting sequence-specific endoribonuclease activity. An example is the hammerhead ribozyme which cleaves at a UH (where H is an A, C or U residue) recognition site in the target RNA and contains base-pairing regions that direct the catalytic domain of the ribozyme to the target site of the substrate RNA. Ribozymes are highly target-specific and can be designed to inactivate one member of a multigene family or targeted to conserved regions of related mRNAs. See, e.g, Merlo et al., The Plant Cell, 10:1603-1621,1998. The ribozyme molecule may be delivered to a cell by transformation, transfection or infection with a vector, such as a plasmid or virus, into which is incorporated the ribozyme operatively

linked to appropriate regulatory elements, including a promoter. Such a ribozyme construct contains base-pairing arms that direct it to a cleavage site within the apoptosis-induced DHS mRNA, or apoptosis-induced eIF-5A mRNA resulting in cleavage of DHS or eIF-5A mRNA and inhibition of apoptosis-induced DHS and/or eIF-5A expression.

"Functional derivatives" of the apoptosis-induced DHS or apoptosis-induced eIF-5A protein as described herein are fragments, variants, analogs, or chemical derivatives of apoptosis-induced DHS or apoptosis-induced eIF-5A, respectively, which retain at least a portion of the apoptosis-induced DHS or eIF-5A activity or immunological cross reactivity with an antibody specific for apoptosis-induced DHS or apoptosis-induced eIF-5A, respectively. A fragment of the apoptosis-induced DHS or apoptosis-induced eIF-5A protein refers to any subset of the molecule. Variant peptides may be made by direct chemical synthesis, for example, using methods well known in the art. An analog of apoptosis-induced DHS or apoptosis-induced eIF-5A refers to a non-natural protein substantially similar to either the entire protein or a fragment thereof. Chemical derivatives of apoptosis-induced DHS or apoptosis-induced -eIF-5A contain additional chemical moieties not normally a part of the peptide or peptide fragment. Modifications may be introduced into peptide or fragment thereof by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues.

Oligonucleotide molecules encoding derivatives of apoptosis-induced eIF-5A that do not up-regulate the expression of apoptosis-induced genes and/ or apoptosis-induced DHS that do not activate eIF-5A may be produced and used to interfere with transcription and/ or translation of endogenous apoptosis-induced eIF-5A and/ or apoptosis-induced

55

DHS. Oligonucleotides encoding such non-functional derivatives of apoptosis-induced eIF-5A and/ or apoptosis-induced DHS proteins may be produced by art-known methods of producing mutations, and their function may be tested in cell-free systems or in cells. Such oligonucleotides may be delivered to a cell by transformation, transfection or infection with a vector, such as a plasmid or virus, into which is incorporated the oligonucleotide, or as naked DNA or DNA associated with liposomes or other delivery systems. Useful oligonucleotides retain enough similarity to the sequences encoding endogenous apoptosis-induced eIF-5A and/ or apoptosis-induced DHS proteins that they knock out the corresponding endogenous gene, or portion thereof, via homologous recombination.

An apoptosis-induced DHS or apoptosis-induced eIF-5A protein or peptide according to the invention may be produced by culturing a cell transformed with a nucleotide sequence of this invention (in the sense orientation), allowing the cell to synthesize the protein and then isolating the protein, either as a free protein or as a fusion protein, depending on the cloning protocol used, from either the culture medium or from cell extracts. Alternatively, the protein can be produced in a cell-free system. Ranu, et al., Meth. Enzymol., 60:459-484, (1979).

In certain preferred embodiments, the present invention employs antisense oligonucleotides, for use in modulating the function of nucleic acid molecules encoding apoptosis-induced DHS and/ or apoptosis-induced eIF-5A, ultimately modulating the amount of apoptosis-induced DHS and/ or apoptosis-induced eIF-5A produced and/ or the amount of activated apoptosis-induced eIF-5A produced. This is accomplished by

providing antisense compounds which specifically hybridize with one or more nucleic acids encoding apoptosis-induced DHS and/ or apoptosis-induced eIF-5A.

The term "antisense oligonucleotide" is intended to refer to nucleic acids, preferably oligonucleotides, that are complementary to the base sequences of a target DNA or RNA. Thus, the term "antisense apoptosis-induced eIF-5A oligonucleotide" refers to oligonucleotides that are complementary to the base sequences of apoptosis-induced eIF-5A DNA or RNA. Similarly, the term "antisense apoptosis-induced DHS oligonucleotide" refers to oligonucleotides that are complementary to the base sequences of apoptosis-induced DHS DNA or RNA. Antisense oligonucleotides, when introduced into a target cell, specifically bind to their target nucleic acid and interfere with transcription, RNA processing, transport, translation and/or stability.

As used herein, the term "antisense oligonucleotide" describes an oligonucleotide that is an oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an mRNA transcript of that gene and, thereby, inhibits the transcription of that gene and/or the translation of that mRNA. Thus, this term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides may be preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases. Modified oligonucleotides, including oligonucleotide mimetics, will be further described herein.

The antisense oligonucleotides of the present invention are designed so as to interfere with transcription or translation of a target gene upon hybridization with the target gene or transcript.

It is preferred to target specific nucleic acids for antisense. Targeting an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, preferred targets are a nucleic acid molecule encoding apoptosis-induced DHS or a nucleic acid molecule encoding apoptosis-induced eIF-5A.

The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., modulation of expression of the protein, will result. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence. It is preferred that the antisense oligonucleotide be constructed and arranged so as to bind selectively with the target under physiological conditions, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions.

Based upon SEQ ID NO: 1 and 3-5 and SEQ ID NO: 6 and 8, or upon allelic or homologous genomic and/or cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance

with the present invention. Antisense nucleotides will bind to a complementary region of a target mRNA and inhibit its translation.

Antisense oligonucleotides are generally 12 to 18 nucleotides in length. In order to be sufficiently selective and potent for inhibition, such antisense oligonucleotides should comprise at least 10 and, more preferably, at least 15 consecutive bases which are complementary to the target, although in certain cases modified oligonucleotides as short as 7 bases in length have been used successfully as antisense oligonucleotides. Wagner et al., Nature Biotechnol. 14:840-844, 1996. Preferably, antisense oligonucleotides according to the present invention comprise a complementary sequence of 16-30 bases.

Although oligonucleotides may be chosen which are antisense to any region of the gene or mRNA transcripts, in certain preferred embodiments the antisense oligonucleotides correspond to N-terminal or 5' upstream sites such as translation initiation, transcription initiation or promoter sites. In addition, 3'-untranslated regions may be targeted by antisense oligonucleotides. Targeting to mRNA splicing sites has also been used in the art but may be less preferred if alternative mRNA splicing occurs. In addition, the antisense is targeted, preferably, to sites in which mRNA secondary structure is not expected and at which proteins are not expected to bind. See, e.g., Sainio et al., Cell Mol. Neurobiol. 14(5):439-457, 1994.

Within the context of the present invention, one preferred intragenic targeting site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the

"AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding DHS or eIF-5A, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Within living cells, mRNA transcripts exist in low energy conformations in which the transcript is highly folded and often interacting with proteins. As a result, only partial sequences within the mRNA transcript are actually available for hybridization. The three-dimension folding pattern of known transcripts can be predicted by RNA folding programs in order to design antisense oligonucleotides against accessible regions of the transcript. For example, the mfold version 3.1 software by Zuker and Turner may be used to predict mRNA secondary structure, accessible portions may be chosen, and antisense nucleotides against these regions may be designed. For additional description of mfold version 3.1 software, see, e.g., Zuker M., et al. "Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide In RNA Biochemistry and Biotechnology, 11-43, J. Barciszewski and B. F. C. Clark, eds., NATO ASI Series, Kluwer Academic Publishers (1999); Mathews DH, et al., "Expanded Sequence Dependence of Thermodynamic Parameters Improves Prediction of RNA Secondary Structure," J. Mol. Biol. 288: 911-40 (1999).

Antisense nucleotides according to the present invention are preferably against portions of the targeted mRNA sequence that are predicted to be accessible. However, in some circumstances, antisense nucleotides against portions of the targeted mRNA sequence that are not predicted to be accessible may be desirable. For example, antisense oligonucleotides directed to a sequence spanning the translation start site of an mRNA molecule have a history of successfully inhibiting translation.

Antisense nucleotides can be tested for effectiveness and efficiency in inhibiting translation by inserting a candidate antisense oligonucleotide into a cell, using art-known methods and methods disclosed herein, and testing for translation of the target protein, again using art-known methods and methods disclosed herein, such as Western blotting with antibodies against the target protein, or portions thereof, and testing for the functionality of the target protein. For example, the efficacy of antisense nucleotides against apoptosis-induced eIF-5A may be tested by measuring apoptosis and/ or the levels of proteins the translation of which is affected by eIF-5A by cells having the candidate antisense oligonucleotide inserted, as compared to cells not having the candidate antisense oligonucleotide inserted. As another example, the efficacy of antisense nucleotides against apoptosis-induced DHS may be tested by measuring apoptosis, levels of activated eIF-5A, and/ or the levels of proteins the translation of which is affected by eIF-5A by cells having the candidate antisense oligonucleotide inserted, as compared to cells not having the candidate antisense oligonucleotide inserted.

Examples of antisense oligonucleotides against rat eIF-5A include the following:

Antisense Oligo 1 : bp 618 to 635 of full-length sequence

       (18 bp) 5' TTG AAG GGG TGA GGA AAA 3' (SEQ ID NO. 19)

Antisense Oligo 2 : bp 845 to 859 of full-length sequence

       (15 bp) 5' TTG AGT GGG ATA AAG 3'   (SEQ ID NO. 20)

Antisense Oligo 3 : spanning ATG start (-5 to + 13)

       (18 bp) 5' AAT CAT CTG CCA TTT TAA 3' (SEQ ID NO. 21).

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

Finally, although, SEQ ID Nos: 1, 3, 4, and 5 and SEQ ID Nos: 6 and 8

disclose cDNA sequences, one of ordinary skill in the art may easily derive the genomic

DNA corresponding to these sequences. Thus, the present invention also provides for

antisense oligonucleotides which are complementary to the genomic DNA corresponding

to SEQ ID NOs: 1, 3, 4, 5 and/or SEQ ID NO: 6 and 8. Similarly, antisense to allelic or

homologous DHS or eIF-5A cDNAs and genomic DNAs are enabled without undue

experimentation.

Antisense compounds in accordance with this invention preferably comprise from

about 8 to about 30 nucleobases (i.e. from about 8 to about 30 linked nucleosides).

Particularly preferred antisense compounds are antisense oligonucleotides, even more

preferably those comprising from about 6 to about 100 nucleobases.

As is known in the art, a nucleoside is a base-sugar combination. The base

portion of the nucleoside is normally a heterocyclic base. The two most common classes

of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are

nucleosides that further include a phosphate group covalently linked to the sugar portion

of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the

phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In

forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to

one another to form a linear polymeric compound. In turn the respective ends of this

linear polymeric structure can be further joined to form a circular structure, however,

open linear structures are generally preferred. Within the oligonucleotide structure, the

phosphate groups are commonly referred to as forming the internucleoside backbone of

the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

In one set of embodiments, the antisense oligonucleotides of the invention may be composed of "natural" deoxyribonucleotides, ribonucleotides, or any combination thereof. That is, the 5' end of one native nucleotide and the 3' end of another native nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleoside linkage. These oligonucleotides may be prepared by art recognized methods which may be carried out manually or by an automated synthesizer. They also may be produced recombinantly by vectors.

However, antisense oligonucleotides containing only phosphodiester linkages may be vulnerable to degradation by nucleases. Thus, in certain preferred embodiments, antisense oligonucleotides of the invention also may include "modified" oligonucleotides. That is, the oligonucleotides may be modified in a number of ways which do not prevent them from hybridizing to their target but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term "modified oligonucleotide" as used herein includes oligonucleotides in which (1) at least two of its nucleotides are covalently linked via a synthetic internucleoside linkage (i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred synthetic internucleoside linkages include phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates,

65

phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, carboxymethyl esters and peptides.

The term "modified oligonucleotide" also encompasses oligonucleotides with a covalently modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-alkylated ribose group. In addition, modified oligonucleotides may include sugars such as arabinose instead of ribose. The present invention, thus, contemplates pharmaceutical preparations containing modified antisense molecules that are complementary to and hybridizable with, under physiological conditions, nucleic acids encoding DHS or eIF-5A polypeptides, together with pharmaceutically acceptable carriers.

To increase stability of the oligonucleotides, they may be synthesized with one or more phosphorothioate linkages in order to decrease degradation by nucleases and to increase the potential for crossing the lipid bilayer. More preferably, the antisense oligonucleotides can be synthesized with two phosphorothioate linkages at both the 5' and 3' ends of the oligonucleotide in order to increase stability.

Specific examples of preferred antisense oligonucleotides useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as

sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

In certain embodiments, one may wish to employ antisense oligonucleotides which include other elements, for example, those which include C-5 propyne pyrimidines. Oligonucleotides which contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression. Wagner et al., "Antisense Gene Inhibition by Oligonucleotides Containing C-5 Propyne Pyrimidines," Science, 260:1510-1513, Jun. 1993.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925;

5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and

5,625,050, each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus

atom therein have backbones that are formed by short chain alkyl or cycloalkyl

internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside

linkages, or one or more short chain heteroatomic or heterocyclic internucleoside

linkages. These include those having morpholino linkages (formed in part from the sugar

portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones;

formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl

backbones; alkene containing backbones; sulfamate backbones; methyleneimino and

methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones;

and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above

oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315;

5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257;

5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289;

5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437;

and 5,677,439, each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the

internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel

groups. The base units are maintained for hybridization with an appropriate nucleic acid

target compound. One such oligomeric compound, an oligonucleotide mimetic that has

been shown to have excellent hybridization properties, is referred to as a peptide nucleic

acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., Science, 1991, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $--CH_2 --NH--O--CH_2 --$, $--CH_2 --N(CH_3)--O--CH_2 --$ [known as a methylene (methylimino) or MMI backbone], $--CH_2 --O--N(CH_3)--CH_2 --$, $--CH_2 --N(CH_3)--N(CH_3)--CH_2 --$ and $--O--N(CH_3)--CH_2 --CH_2 --$ [wherein the native phosphodiester backbone is represented as $--O--P--O--CH_2 --$] of the above referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above referenced U.S. Pat. No. 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_n O]_m CH_3$, $O(CH_2)_n OCH_3$, $O(CH_2)_n NH_2$, $O(CH_2)_n CH_3$, $O(CH_2) ONH_2$, and $O(CH_2)_n ON$

$[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O--CH_2 CH_2OCH_3, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., Helv. Chim. Acta, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2$ $ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'--O--CH_2--O--CH_2 --$N(CH_2)_2$.

Other preferred modifications include 2'-methoxy (2'-O--CH_3), 2'-aminopropoxy (2'-OCH_2 CH_2 CH_2 NH_2) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786;

5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, each of which is herein incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., Angewandte Chemie, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y. S., Chapter 15, Antisense Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B. ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the

invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and

O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-

propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic

acid duplex stability by 0.6-1.2 °C. (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., eds.,

Antisense Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and

are presently preferred base substitutions, even more particularly when combined with 2'-

O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the

above noted modified nucleobases as well as other modified nucleobases include, but are

not limited to, the above noted U.S. Pat. Nos. 3,687,808, as well as U.S. Pat. Nos.

4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187;

5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091;

5,614,617; and 5,681,941, each of which is herein incorporated by reference, and United

States patent 5,750,692, which is commonly owned with the instant application and also

herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically

linking to the oligonucleotide one or more moieties or conjugates which enhance the

activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties

include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al.,

Proc. Natl. Acad. Sci. USA, 1989, 86, 6553-6556), cholic acid (Manoharan et al., Bioorg.

Med. Chem. Let., 1994, 4, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et

al., Ann. N.Y. Acad. Sci., 1992, 660, 306-309; Manoharan et al., Bioorg. Med. Chem.

Let., 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., Nucl. Acids Res., 1992,

20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., EMBO J., 1991, 10, 1111-1118; Kabanov et al., FEBS Lett., 1990, 259, 327-330; Svinarchuk et al., Biochimie, 1993, 75, 49-54), a phospholipid, e.g. di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654; Shea et al., Nucl. Acids Res., 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., Nucleosides & Nucleotides, 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., Biochim. Biophys. Acta, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther., 1996, 277, 923-937.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a

single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense oligonucleotides which are chimeric compounds. "Chimeric" antisense oligonucleotides or "chimeras," in the context of this invention, are antisense oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, e.g., a nucleotide in the case of an oligonucleotide. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense oligonucleotides of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S. Pat.

Nos. 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, each of which is herein incorporated by reference in its entirety.

The antisense oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Nucleic acids of any desired sequence may be sythesized using, e.g., automated synthesis machines, which are commercially available. Examples of such machines include the Expedite™ 8909 Nucleic Acid Synthesizer from Applied BioSystems and the ÄKTA OligoPilot DNA/ RNA Synthesizer from Amersham Pharmacia Biotech.

Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense oligonucleotides used in accordance with this invention may also be made through the use of an expression construct. Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. Thus, in certain embodiments, expression includes both transcription of a gene and translation of a RNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid, for example, to generate antisense oligonucleotides. Expression constructs can be used to produce gene products,

such as mRNA and proteins, *in vitro* in cell culture, and can also be used to produce gene

products *ex vivo* and *in vivo*, making them useful for e.g. gene therapy applications.

For *in vitro* production, cell culture techniques are well documented in the art and

are disclosed herein by reference. Freshner, "Animal Cell Culture: A Practical

Approach," Second Edition, Oxford/New York, IRL Press, Oxford University Press,

1992.

Expression constructs containing all the necessary elements for expression are

commercially available and known to those skilled in the art. See, e.g., Sambrook et al.,

Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor

Laboratory Press, 1989. Cells are genetically engineered by the introduction into the

cells of heterologous DNA (or RNA) encoding DHS and/ or eIF-5A polypeptide or

fragment or variant thereof. That heterologous DNA/ RNA is placed under operable

control of transcriptional elements to permit the expression of the heterologous DNA in

the host cell.

Preferred systems for mRNA expression in mammalian cells are those such as

pRc/CMV (available from Invitrogen, Carlsbad, Calif.) that contain a selectable marker

such as a gene that confers G418 resistance (which facilitates the selection of stably

transfected cell lines) and the human cytomegalovirus (CMV) enhancer-promoter

sequences. Additionally, suitable for expression in primate or canine cell lines is the

pCEP4 vector (Invitrogen), which contains an Epstein Barr virus (EBV) origin of

replication, facilitating the maintenance of plasmid as a multicopy extrachromosomal

element. Another expression vector is the pEF-BOS plasmid containing the promoter of

polypeptide Elongation Factor 1α, which stimulates efficiently transcription *in vitro*. The

plasmid is described by Mishizuma and Nagata (Nuc. Acids Res. 18:5322, 1990), and its use in transfection experiments is disclosed by, for example, Demoulin (Mol. Cell. Biol. 16:4710-4716, 1996). Still another preferred expression vector is an adenovirus, described by Stratford-Perricaudet, which is defective for E1 and E3 proteins (J. Clin. Invest. 90:626-630, 1992). The use of the adenovirus as an Adeno.P1A recombinant is disclosed by Warnier et al., in intradermal injection in mice for immunization against P1A (Int. J. Cancer, 67:303-310, 1996).

The invention also embraces "expression kits", which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of each of the previously discussed coding sequences. Other components may be added, as desired, as long as the previously mentioned sequences, which are required, are included.

In preferred embodiments, the nucleic acid is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that

77

promoters are composed of discrete functional modules, each consisting of approximately

7-20 bp of DNA, and containing one or more recognition sites for transcriptional

activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA

synthesis. The best known example of this is the TATA box, but in some promoters

lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl

transferase gene and the promoter for the SV40 late genes, a discrete element overlying

the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation.

Typically, these are located in the region 30-110 bp upstream of the start site, although a

number of promoters have recently been shown to contain functional elements

downstream of the start site as well. The spacing between promoter elements frequently

is flexible, so that promoter function is preserved when elements are inverted or moved

relative to one another. In the tk promoter, the spacing between promoter elements can

be increased to 50 bp apart before activity begins to decline. Depending on the promoter,

it appears that individual elements can function either co-operatively or independently to

activate transcription.

The particular promoter that is employed to control the expression of a nucleic

acid is not believed to be critical, so long as it is capable of expressing the nucleic acid in

the targeted cell. Thus, where a human cell is targeted, it is preferable to position the

nucleic acid coding region adjacent to and under the control of a promoter that is capable

of being expressed in a human cell: Generally speaking, such a promoter might include

either a human or viral promoter. For example, promoters include those derived from

HSV, including the α4 promoter. Another exemplary promoter is the tetracycline controlled promoter.

In various other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of transgenes. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a transgene is contemplated as well, provided that the levels of expression are sufficient for a given purpose.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of a transgene. Use of a T3, T7

or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells

can support cytoplasmic transcription from certain bacterial promoters if the appropriate

bacterial polymerase is provided, either as part of the delivery complex or as an

additional genetic expression construct.

A baculovirus system, which would involve high level expression from the

powerful polyhedron promoter, may also be used for expression of antisense

oligonucleotides *in vitro*.

One will typically include a polyadenylation signal to effect proper

polyadenylation of the transcript. The nature of the polyadenylation signal is not

believed to be crucial to the successful practice of the invention, and any such sequence

may be employed. Preferred embodiments include the SV40 polyadenylation signal and

the bovine growth hormone polyadenylation signal, convenient and known to function

well in various target cells. Also contemplated as an element of the expression cassette is

a terminator. These elements can serve to enhance message levels and to minimize read

through from the cassette into other sequences.

A specific initiation signal also may be required for efficient translation of coding

sequences. These signals include the ATG initiation codon and adjacent sequences.

Exogenous translational control signals, including the ATG initiation codon, may need to

be provided. One of ordinary skill in the art would readily be capable of determining this

and providing the necessary signals. It is well known that the initiation codon must be

"in-frame" with the reading frame of the desired coding sequence to ensure translation of

the entire insert. The exogenous translational control signals and initiation codons can be

either natural or synthetic. The efficiency of expression may be enhanced by the

inclusion of appropriate transcription enhancer elements. Bittner et al., Methods in Enzym, 153:516-544, 1987.

Expression constructs may be replicated by, e.g., the use of a cloning vector. A cloning vector is one which is able to replicate in a host cell, and which is further characterized by one or more endonuclease restriction sites at which the vector may be cut in a determinable fashion and into which a desired DNA sequence may be ligated such that the new recombinant vector retains its ability to replicate in the host cell. In the case of plasmids, replication of the desired sequence may occur many times as the plasmid increases in copy number within the host bacterium or just a single time per host before the host reproduces by mitosis. In the case of phage, replication may occur actively during a lytic phase or passively during a lysogenic phase.

The antisense oligonucleotides of the invention may be synthesized *in vitro* or may be produced in vivo via genetic vector constructs designed to direct the *in vivo* synthesis of antisense molecules.

In order to effect expression of constructs encoding genes of interest, the expression construct must be delivered into a cell. The cell can be *in vitro*, *ex vivo*, or *in vivo*.

Mechanisms for delivery that may be used to transfer expression vectors into cells of interest include vectors such as plasmids, phagemids, and viral systems.

One mechanism for delivery is via viral infection, where the expression construct is encapsidated in a viral particle which will deliver either a replicating or non-replicating nucleic acid. In certain embodiments, the preferred vector is an HSV vector, although virtually any vector would suffice, and vectors known to be more or less appropriate for

different purposes are known to the art. Similarly, where viral vectors are used to deliver other therapeutic genes, inclusion in these vectors of a gene or genes according to the present invention advantageously will protect the cell from virally induced apoptosis, thereby facilitating gene therapy using other gene constructs.

The ability of certain viruses to enter cells via receptor-mediated endocytosis and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells. Ridgway, In: Vectors, A Survey of Molecular Cloning Vectors and Their Uses, Rodriquez and Denhardt, (Eds.), Boston: Butterworths, Chapter 24:467-492, 1988; Nicolas and Rubenstein, In: Vectors, A Survey of Molecular Cloning Vectors and Their Uses, Rodriguez and Denhardt (Eds.), Boston: Butterworths, Chapter 25:493-513, 1988; Baichwal and Sugden, In: GENE TRANSFER Kucherlapati, R., ed. New York: Plenum Press, pp. 117-148, 1986; Temin, In: Gene Transfer, Kucherlapati (Ed.), New York: Plenum Press, Chapter 6:149-188, 1986. The first viruses used as vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma). Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986) and adeno-associated viruses. Retroviruses also are attractive gene transfer vehicles (Nicolas and Rubenstein, 1988; Temin, 1986) as are vaccina virus (Ridgeway, 1988) and adeno-associated virus (Ridgeway, 1988). Such vectors may be used to (i) transform cell lines *in vitro* for the purpose of producing mRNA and/ or proteins of interest or (ii) to transform cells *in vitro* or *in vivo* to provide therapeutic mRNA and/ or polypeptides in a gene therapy scenario.

In one embodiment, the vector is HSV. Because HSV is neurotropic, it has generated considerable interest in treating nervous system disorders. Moreover, the ability of HSV to establish latent infections in non-dividing neuronal cells without integrating in to the host cell chromosome or otherwise altering the host cell's metabolism, along with the existence of a promoter that is active during latency, make it useful as a vector. And though much attention has focused on the neurotropic applications of HSV, this vector also can be exploited for other tissues.

Another factor that makes HSV an attractive vector is the size and organization of the genome. Because HSV is large, incorporation of multiple genes or expression cassettes is less problematic than in other smaller viral systems. In addition, the availability of different viral control sequences with varying performance (temporal, strength, etc.) makes it possible to control expression to a greater extent than in other systems. It also is an advantage that the virus has relatively few spliced messages, further easing genetic manipulations.

HSV also is relatively easy to manipulate and can be grown to high titers. Thus, delivery is less of a problem, both in terms of volumes needed to attain sufficient MOI and in a lessened need for repeat dosings. For a review of HSV as a gene therapy vector, see Glorioso et al., Annu Rev Microbiol, 49:675-710, 1995.

Adenovirus, adeno-associated virus, herpes virus, vacciniavirus, retroviruses, or other viral vectors may be used as a gene transfer delivery system for a therapeutic genetic construct. Viral vectors which do not require that the target cell be actively dividing, such as adenoviral and adeno-associated vectors, are particularly useful when the target cells are not proliferative. Numerous vectors useful for this purpose are

generally known. See, e.g., Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244: 1275-1281, 1989; Eglitis and Anderson, BioTechniques 6: 608-614, 1988; Tolstoshev and Anderson, Current Opinion in Biotechnology 1: 55-61, 1990; Sharp, The Lancet 337: 1277-1278, 1991; Cornetta et al., Nucleic Acid Research and Molecular Biology 36: 311-322, 1987; Anderson, Science 226: 401-409, 1984; Moen, Blood Cells 17: 407-416, 1991; and Miller and Rosman, Bio Techniques 7: 980-990, 1989; Le Gal La Salle et al., Science 259: 988-990, 1993; and Johnson, Chest 107: 77S-83S, 1995). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg et al., N. Engl. J. Med 323: 370, 1990; Anderson et al., U.S. Pat. No. 5,399,346.

A viral vector can be made target specific by inserting an apoptosis-regulating gene sequence of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell. Viral vectors can be made target specific by attaching, for example, a sugar, a glycolipid, or a protein. Targeting may also be accomplished by using an antibody to target the viral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the viral genome or attached to a viral envelope to allow target specific delivery of viral vectors.

Since recombinant viruses used as vectors are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids encoding all of the structural genes of the virus under the control of regulatory sequences within the viral genome. These plasmids are missing a nucleotide sequence which enables the packaging mechanism to recognize

84

a polynucleotide transcript for encapsidation. These cell lines produce empty virons,

since no genome is packaged. If a viral vector is introduced into such cells in which the

packaging signal is intact, but the structural genes are replaced by other genes of interest,

the vector can be packaged and vector virion produced.

For example, using the methods of the invention, an adenovirus vector [which

may be obtained from, e.g., Microbix Biosystems Inc. (Toronto, Canada)] can be used to

achieve transient expression of the apoptosis-induced eIF-5A nucleotide sequence,

fragments thereof, and/ or combinations of such fragment, or the apoptosis-induced DHS

nucleotide sequence, fragments thereof, or combinations of such fragment in the

antisense orientation in animal/human cells and tissues. Adenoviruses are widely used

for transient expression of DNA in both cultured cells and animals due to their ability to

infect a wide range of cell types. One example of an appropriate adenovirus vector

contains deletions in the E1 and E3 regions to render the adenovirus non-infectious

except when introduced into the E1-complementing 293 cells used for propagation of the

virus. The adenovirus vector also expresses FLP recombinase to allow introduction of

desired DNA by site-specific recombination between the adenovirus and a shuttle vector

carrying the DNA of interest. The DNA to be expressed is subcloned downstream of the

constitutively expressed murine cytomegalovirus immediate early gene promoter in the

pDC516 shuttle vector provided with the adenovirus construction kit. The shuttle vector

containing the DNA to be expressed is then cotransfected with the adenovirus vector,

pBHGfrtΔE1,3FLP, into the E1-complementing 293 cell line. Cotransfection of these

two plasmids introduces the promoter and the DNA to be expressed into the adenovirus

vector while simultaneously removing the recombinase gene. The adenovirus vector is

replication-competent in the E1-complementing cells, and plaques containing

recombinant adenovirus vector can be isolated and purified by cesium chloride banding

approximately 10 days after cotransfection.

Recombinant adenovirus can be delivered to cultured cells by simply adding the

virus to the culture media. Infection of host animals/humans can be achieved by directly

injecting the viral particles into the bloodstream or into the desired tissue. The half-life

of the virus in serum can be extended by complexing the virus with liposomes (e.g.

Lipofectin, Life Technologies) or polyethylene glycol. The adenovirus vector normally

enters the cell through an interaction between the knob domain of the viral fiber protein

and the coxsackievirus and adenovirus receptor, CAR. The viral vector can be directed to

specific cells, or to cells which do not express the CAR, by genetically engineering the

virus to express a ligand specific to a certain cell receptor.

Non-viral approaches may also be employed for the introduction polynucleotides,

e.g., antisense polynucleotides, into cells otherwise predicted to undergo or undergoing

excessive, premature, and/ or inappropriate apoptosis. For example, an antisense

polynucleotide may be introduced into target cell by the techniques of colloidal

dispersion; asialorosonucoid-polylysine conjugation; or microinjection under surgical

conditions. See, e.g., Felgner et al., Proc. Natl. Acad Sci. USA 84: 7413, 1987; Ono et

al., Neuroscience Lett 117: 259, 1990; Brigham et al., Am. J Med. Sci. 298: 278, 1989;

Staubinger and Papahadjopoulos, Meth. Enz. 101: 512, 1983; Wu and Wu, J. Biol.

Chem. 263: 14621, 1988; Wu et al., J. Biol. Chem. 264: 16985, 1989; Wolff et al.,

Science 247: 1465, 1990. Additional examples include calcium phosphate precipitation,

DEAE-dextran, electroporation, direct microinjection, DNA-loaded liposomes and

lipofectamine-DNA complexes, cell sonication, gene bombardment using high velocity

microprojectiles, and receptor-mediated transfection. See, e.g., Graham and Van Der Eb,

Virology, 52:456-467, 1973 (phosphate precipitation); Chen and Okayama, Molecular

and Cellular Biology, 7(8):2745-2752, 1987 (phosphate precipitation); Rippe et al., Mol

Cell Biol, 10(2):689-695, 1990 (phosphate precipitation); Gopal, Molecular and Cellular

Biology, 5(5):1188-1190, 1985 (DEAE-dextran); Tur-Kaspa et al., Mo. Cell. Biol.

6(2):716-718, 1986 (electroporation); Potter et al., Proc. Natl. Acad. Sci., USA, 81:7161-

7165, 1984 (electroporation); Harland and Weintraub, J Cell Biol, 101:1094-1099, 1985

(direct microinjection); Nicolau and Sene, Biochimica et Biophysica Acta, 721:185-190,

1982 (DNA-loaded liposomes and lipofectamine-DNA complexes); Fraley et al., Proc.

Natl. Acad. Sci., USA, 76(7):3348-3352, 1979 (DNA-loaded liposomes and

lipofectamine-DNA complexes); Fechheimer et al., Proc. Natl. Acad. Sci., USA,

84:8463-8467, 1987 (cell sonication); Yang et al., Proc. Natl. Acad. Sci., USA,

87:9568-9572, 1990 (gene bombardment using high velocity microprojectiles); Wu and

Wu, J Biol Chem, 262(10):4429-4432, 1987 (receptor-mediated transfection); and Wu

and Wu, Biochemistry, 27:887-892, 1988 (receptor-mediated transfection).

In another embodiment of the invention, the expression construct may simply

consist of naked recombinant DNA or plasmids. Transfer of the construct may be

performed by any of the methods discussed herein which physically or chemically

permeabilize the cell membrane. This is particularly applicable for transfer *in vitro*, but it

may be applied to *in vivo* use as well. Another embodiment of the invention for

transferring a naked DNA expression construct into cells may involve particle

bombardment. This method depends on the ability to accelerate DNA coated

microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them. Klein et al., Nature, 327:70-73, 1987. Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force. Yang et al., Proc. Nat'l Acad. Sci. USA, 87:9568-9572, 1990. The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. These artificial membrane vesicles are useful as delivery vehicles *in vitro* and *in vivo*. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers. Ghosh and Bachhawat, In: Liver Diseases, Targeted Diagnosis and Therapy Using Specific Receptors and Ligands, Wu and Wu (Eds.), New York:Marcel Dekker, Inc., pp. 87-104, 1991. Also contemplated are lipofectamine-DNA complexes.

It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μm, can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virons can be encapsulated within the

aqueous interior and be delivered to cells in a biologically active form. Fraley, et al., Trends Biochem. Sci., 6: 77, 1981. In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should preferably be present: encapsulation of the genes of interest at high efficiency while not compromising their biological activity; preferential and substantial binding to a target cell in comparison to non-target cells; delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (Kinoshita, et al., supra) accurate and effective expression of genetic information (Mannino, et al., Bio Techniques, 6: 682, 1988). See, e.g., Zakut and Givol, Oncogene, 11(2): 393, 1995; Fearnhead, et al., Febs Letter, 375(2): 303, 1995; Korsmeyer, S. J., Trends in Genetics, 11(3):101, 1995; Kinoshita, et al., Oncogene, 10(11): 2207, 1995. Additional discussion of the production and use of liposomes is provided herein.

The composition of the liposome is usually a combination of phospholipid, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations. The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active

targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Wong et al. demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa, and hepatoma cells. Wong, et al., Gene, 10 (1980) 87-94. In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). Such complexing has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA. Kaneda et al., Science, 243:375-378, Jan. 1989. In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1). Kato et al., J Biol Chem, 266(6):3361-3364, Feb. 1991. In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In other embodiments, the delivery vehicle may comprise a ligand and a liposome. Where a bacterial promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacterial polymerase.

Other expression constructs which can be employed to deliver genes of interest into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can

be highly specific. Wu and Wu, Advanced Drug delivery Reviews, 12: 159-167 (1993) (delivery to liver).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) and transferrin. Wu and Wu (1987) (ASOR); Wagner et al., Science, 260:1510-1513, 1990 (transferrin). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle. Ferkol et al., FASEB Journal, 7:1081-1091, 1993; Perales et al., Proc. Natl. Acad. Sci., USA, 91:4086-4090, 1994. Mannose can be used to target the mannose receptor on liver cells. Also, antibodies to CD5 (CLL), CD22 (lymphoma), CD25 (T-cell leukemia) and MAA (melanoma) can similarly be used as targeting moieties. In other embodiments, the delivery vehicle may comprise a ligand and a liposome.

Gene therapy may also be accomplished by direct administration of the apoptosis-regulating polynucleotide to a cell. The polynucleotide may be produced and isolated by any standard technique. For example, mRNA is most readily produced by *in vitro* transcription using a cDNA under the control of a high efficiency promoter (e.g., the T7 promoter). Administration of mRNA to accumulated cells is carried out by any of the methods for direct nucleic acid administration described herein and known in the art.

Many of the gene transfer techniques that generally are applied *in vitro* have been or can be adapted for *ex vivo* or *in vivo* use. For example, selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo*. Yang et al., 1990; Zelenin et al., FEBS Lett., 280:94-96, 1991. Naked DNA also has been used in

clinical settings to effect gene therapy. These approaches may require surgical exposure of the target tissue or direct injection into the target tissue. Nicolau et al. (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection. Nicolau et al., Methods Enzymol., 149:157-176, 1987.

Dubensky et al. (1984) successfully injected polyomavirus DNA in the form of $CaPO_4$ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Dubensky et al,. Proc. Nat'l Acad. Sci. USA, 81:7529-7533, 1984. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of $CaPO_4$ precipitated plasmids results in expression of the transfected genes. Benvenisty & Neshif, Proc. Nat'l Acad. Sci. USA 83:9551-9555, 1986. Thus, it is envisioned that DNA encoding an antisense oligonucleotides also may be transferred in a similar manner *in vivo*.

As another example, a procedure for performing *ex vivo* gene therapy is outlined in U.S. Pat. No. 5,399,346 and in exhibits submitted in the file history of that patent, all of which are publicly available documents. In general, it involves introduction *in vitro* of a functional copy of a gene into a cell(s) of a subject which contains a defective copy of the gene, and returning the genetically engineered cell(s) to the subject. The functional copy of the gene is under operable control of regulatory elements which permit expression of the gene in the genetically engineered cell(s). Numerous transfection and transduction techniques as well as appropriate expression vectors are well known to those of ordinary skill in the art, some of which are described in PCT application WO95/00654. *In vivo* gene therapy using vectors such as adenovirus, retroviruses, herpes virus, and targeted liposomes also is contemplated according to the invention. Methods for *in vivo*

gene therapy are known in the art. For example, viral means of introducing DNA into mammalian cells are known in the art. In particular, a number of vector systems are known for the introduction of foreign or native genes into mammalian cells. These include SV40 virus (See, e.g., Okayama et al. (1985) Molec. Cell Biol. 5:1136-1142); Bovine papilloma virus (See, e.g., DiMaio et al. (1982) Proc. Natl. Acad. Sci. USA 79:4030-4034); adenovirus (See, e.g., Morin et al. (1987) Proc. Natl. Acad. Sci. USA 84:4626; is Yifan et al. (1995) Proc. Natl. Acad. Sci. USA 92:1401-1405; Yang et al. (1996) Gene Ther. 3:137-144; Tripathy et al. (1996) Nat. Med. 2:545-550; Quantin et al. (1992) Proc. Natl. Acad. Sci. USA 89:2581-2584; Rosenfeld et al. (1991) Science 252:431-434; Wagner (1992) Proc. Nati. Acad. Sci. USA 89:6099-6103; Curiel et al. (1992) Human Gene Therapy 3:147-154; Curiel (1991) Proc. Natl. Acad. Sci. USA 88:8850-8854; LeGal LaSalle et al. (1993) Science 259:590-599); Kass-Eisler et al. (1993) Proc. Nati. Acad. Sci. USA 90:11498-11502); adeno-associated virus (See, e.g., Muzyczka et al. (1994) J. Clin. Invest. 94:1351; Xiao et al. (1996) J. Virol. 70:8098-8108); herpes simplex virus (See, e.g., Geller et al. (1988) Science 241:1667; Huard et al. (1995) Gene Therapy 2:385-392; U.S. Pat. No. 5,501,979); retrovirus-based vectors (See, for example, Curran et al. (1982) J. Virol. 44:674-682; Gazit et al. (1986) J. Virol. 60:19-28; Miller, A.D. (1992) Curr. Top. Microbiol. Immunol. 158:1-24; Cavanaugh et al. (1994) Proc. Natl. Acad. Sci. USA 91:7071-7075; Smith et al. (1990) Molecular and Cellular Biology 10:3268-3271); herein incorporated by reference. See also, Wu et al. (1991) J. Biol. Chem. 266:14338-14342; Wu and Wu (J. Biol Chem. (1988)) 263:14621-14624; Wu et al. (1989) J. Biol. Chem. 264:16985-16987; Zenke et al. (1990) Proc. Natl.

93

Acad. Sci. USA 87:3655-3659; Wagner et al. (1990) 87:3410-3414; Jolly, Cancer Gene Therapy (1994) 1:51-64.

For any of the above approaches, therapeutic antisense polynucleotide constructs and/ or other therapeutic agents are preferably applied to the site where reduction in apoptosis is desirable (e.g., by injection), but may also be applied to tissue in the vicinity of the needed modulation of apoptosis, or, for example, to a blood vessel supplying the cells in which modulation of apoptosis is desirable. Additionally, therapeutic antisense polynucleotide constructs and/ or other therapeutic agents may be administered systemically.

In the gene delivery constructs of the instant invention, poly- or oligonucleotide expression may be directed from any suitable promoter (e.g., the human cytomegalovirus, simian virus 40, actin or adenovirus constitutive promoters; or the cytokine or metalloprotease promoters for activated synoviocyte specific expression). Furthermore, poly- or oligonucleotide production may be regulated by any desired mammalian regulatory element or other appropriate regulatory element. Such enhancers include, without limitation, those enhancers which are characterized as tissue or cell specific in their expression.

Drugs, chemicals, liposomes, vectors, naked oligonucleotides, and other compositions according to the present invention mat be formulated with pharmaceutically acceptable carriers for administration *in vivo, ex vivo,* or *in vitro.*

The compositions of this invention may be in a variety of forms. These include, for example, solid, semi-solid and liquid dosage forms, such as tablets, pills, powders, liquid solutions, dispersions or suspensions, liposomes, suppositories, injectable and

infusible solutions. The preferred form depends on the intended mode of administration and therapeutic application. The preferred compositions are in the form of injectable or infusible solutions.

Effective dosages and scheduling regimens of administration of antibodies according to the present invention can be determined by the skilled practitioner using art-known methods, such as clinical trials and animal studies. Concentrations of the administered substances will vary depending upon the therapeutic or preventive purpose.

Formulations according to the present invention may be administered orally, intravenously, intramuscularly, subcutaneously, topically, intratracheally, intrathecally, intraperitoneally, rectally, vaginally, intracerebrospinally, via inhalation, topically, or intrapleurally. A preferred mode of administration is intraperitoneal injection. Another preferred mode of delivery is injection at the site of, near, or into the vasculature surrounding target cells. Yet another preferred mode of delivery is placement of a sustained release or other implant at the site of or near target cells.

If formulations according to the present invention are administered orally, they may be administered in the form of a tablet, a pill, a liquid or a capsule. Liquid formulations comprising compounds according to the present invention may also be formulated as sprays, which may be formulated to be suitable for, e.g., spraying into the mouth or spraying onto a wound and/ or the surrounding area.

Compositions according to the present invention may be administered as buccal, lingual, or sublingual tablets, capsules, or lozenges.

A liquid may be administered in the form of a solution or a suspension. The liquid dosage form may comprise, for example, an alcohol-base or may be formulated with a cyclodextrin such as hydroxypropyl-β-cyclodextrin.

Forulations produced in accordance with the invention may comprise conventional pharmaceutically acceptable diluents or carriers. Tablets, pills, liquids and capsules may include conventional excipients such as lactose, starch, cellulose derivatives, hydroxypropyl methylcellulose and magnesium stearate. Conventional enteric coatings may also be used.

Dosage forms such as oral, rectal, vaginal, and internal implants may be formulated for immediate release or for delayed release. Likewise, such dosage forms may be in a sustained-release formulation or in a once a day formulation.

Suppositories may include excipients such as waxes and glycerol. Injectable solutions will comprise sterile pyrogen-free media such as saline and may include buffering agents, stabilizing agents, solubilizing agents or preservatives.

Compositions for topical administration may be in the form of creams, ointments, lotions, solutions, transdermal delivery systems, transdermal patches, foams, or gels.

All citations throughout the specification and the references cited therein are hereby expressly incorporated by reference.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration. The Examples are set forth to aid in understanding the invention but are not intended to, and should not be construed to, limit its scope in any way. The examples do

not include detailed descriptions of conventional methods. Such methods are well known to those of ordinary skill in the art and are described in numerous publications.

Example 1

Superovulation and Induction of Apoptosis in Rat Corpus Luteum

Immature (21-30 day old) female rats were superovulated by subcutaneous injection with 50 IU of PMSG (Pregant Mare Serum Gonadotropin) and, 60 to 65 hours later, with 50 IU of HCG (Human Chorionic Gonadotropin). Seven days after the treatment with HCG, corpus luteum apoptosis was induced by subcutaneous injection with 500 µg of $PGF_{2\alpha}$ (Prostaglandin $F_{2\alpha}$). Rats were sacrificed at various times (e.g 1, 8 and 24 hours) after $PGF_{2\alpha}$ treatment, and the corpora lutea were removed and placed in liquid nitrogen. Control corpus luteum tissue was obtained by sacrificing rats immediately before $PGF_{2\alpha}$ treatment.

Dispersion of Rat Ovarian Corpus Luteum Cells

Six to nine days after superovulation, rats were treated by multisite subcutaneous injection with 500 µg prostaglandin $F_{2\alpha}$. Fifteen to thirty minutes later, the ovaries were removed from the superovulated rats, placed in EBSS (Gibco) on ice, blotted dry and weighed. Connective tissue was trimmed away, and the ovaries were minced finely with a razor blade and washed twice with EBSS 2X. Collagenase solution was prepared by vortexing 6.5 mg of collagenase (Sigma, Catologue # C 5138) in 5ml of EBSS. Minced tissue from 8 ovaries was added to 5 ml of collagenase in EBSS in a 50 ml Erlenmeyer flask and agitated gently by withdrawing several times into a Diamed pipette. The flask with minced tissue was then placed in a water bath at 37°C for 20 minutes with gentle

shaking (Position 45 on GFL incubator) under 95% air : 5% CO_2. Following this

incubation, the flask was placed on ice, and the suspension of cells was transferred with a

plastic transfer pipet onto a Nitex filter fitted with Swiss Nitex Nylon Monofilament (75

μ). The filtrate was collected into a 15 ml Falcon test tube. A second aliquot (2.5 ml) of

collagenase solution (6.5 mg collagenase/5ml EBSS) was added to the minced tissue

remaining in the 50 ml Erlenmeyer flask, agitated gently using a pipette, incubated for 10

minutes and filtered as above. The two filtrates were combined and centrifuged in a

clinical centrifuge(~200g) for 5 minutes at room temperature. All but ~2ml of the

supernatant were removed with a pipet and discarded, and the sedimented cells were

resuspended in the remaining 2 ml of supernatant. The cells were washed twice by adding

5 ml of MEM and centrifuging and resuspending as above. The washed cells were

resuspended in 30 mls of MEM containing 10 mM glutamine in a 50 ml Erlenmeyer

flask and incubated for 1 hour without shaking at 37° C under 95% air, 5% CO_2. The

cells were then sedimented by centrifugation as above and resuspended in MEM

containing 10 mM glutamine. The concentration of dispersed cells was determined using

a hemocytometer, and viability was assessed using trypan blue dye. Aliquots of 2-5 x10^5

cells were placed in 12x75 mm test tubes and incubated without shaking for 2-5 hours at

37° C under 95% air: % CO_2. The progress of apoptosis during this period was monitored

by assessing the degree of DNA laddering.

Visualization of Apoptosis in Rat Corpus Luteum by DNA Laddering

The degree of apoptosis was determined by DNA laddering. Genomic DNA was

isolated from dispersed corpus luteal cells or from excised corpus luteum tissue using the

QIAamp DNA Blood Kit (Qiagen) according to the manufacturer's instructions. Corpus

luteum tissue was excised before the induction of apoptosis by treatment with

prostaglandin $F_{2\alpha}$ as well as 1 hour and 24 hours after induction of apoptosis. The

isolated DNA was end-labeled by incubating 500 ng of DNA with 0.2 µCi [α-^{32}P]dCTP,

1 mM Tris, 0.5 mM EDTA, 3 units of Klenow enzyme, and 0.2 pM each of dATP, dGTP,

and dTTP, at room temperature for 30 minutes. Unincorporated nucleotides were

removed by passing the sample through a 1-ml Sephadex G-50 column according to the

method described by Maniatis *et al.*. The samples were then resolved by Tris-acetate-

EDTA (1.8 %) gel electrophoresis. The gel was dried for 30 minutes at room

temperature under vacuum and exposed to x-ray film at - 80°C for 24 hours.

Laddering of low molcular weight DNA fragments reflecting nuclease activity

associated with apoptosis is not evident in control corupus luteum tissue excised before

treatment with prostaglandin $F_{2\alpha}$, but is discernible within 1 hour after induction of

apoptosis and is pronounced by 24 hours after induction of apoptosis (FIG. 19). DNA

laddering reflecting apoptosis is also evident when corpora lutea are excised 15 minutes

after treatment with prostaglandin $F_{2\alpha}$ and maintained for 6 hours under *in vitro*

conditions in EBSS (Gibco) (FIG. 20). Nuclease activity associated with apoptosis is also

evident from more extensive end-labelling of genomic DNA (FIGS. 20 and 19).

Example 2

RNA Isolation

Total RNA was isolated from corpus luteum tissue removed from rats at various

times after PGF$_{2\alpha}$ induction of apoptosis. Briefly, the tissue (5 g) was ground in liquid

nitrogen. The ground powder was mixed with 30 ml guanidinium buffer (4 M

guanidinium isothiocyanate, 2.5 mM NaOAc pH 8.5, 0.8% β-mercaptoethanol). The mixture was filtered through four layers of Miracloth and centrifuged at 10,000 Xg at 4°C for 30 minutes. The supernatant was then subjected to cesium chloride density gradient centrifugation at 11,200g for 20 hours. The pelleted RNA was rinsed with 75% ethanol, resuspended in 600 μl DEPC-treated water and the RNA precipitated at -70°C with 1.5 ml 95% ethanol and 60 μl of 3M NaOAc. Twenty μg of RNA were fractionated on a 1.2% denaturing formaldehyde agarose gel and transferred to a nylon membrane. Randomly primed 32p-dCTP-labeled full length eIF-5A cDNA (SEO ID NO:1) or partial length DHS cDNA (SEQ ID NO. 6) were used to probe the membrane at 42°C overnight. The membrane was then washed once in 1 X SSC containing 0.1% SDS at room temperature for 15 minutes and three times in 0.2X SSC containing 0.1% SDS at 65°C for 15 minutes each. The membrane was exposed to X-ray film overnight at -70°C.

Genomic DNA Isolation and Laddering

Genomic DNA was isolated from extracted corpus luteum tissue or dispersed corpus luteal cells using the QIAamp DNA Blood Kit (Qiagen) according to the manufacturer's instructions. The DNA was end-labeled by incubating 500 ng of DNA with 0.2 μCi [α-^{32}P]dCTP, 1 mM Tris, 0.5 mM EDTA, 3 units of Klenow enzyme, and 0.2 pM each of dATP, dGTP, and dTTP, at room temperature for 30 minutes. Unincorporated nucleotides were removed by passing the sample through a 1-ml Sephadex G-50 column according to the method described by Maniatis *et al.* The samples were then resolved by Tris-acetate-EDTA (2 %) gel electrophoresis. The gel was dried for 30 minutes at room temperature under vacuum and exposed to x-ray film at - 80°C for 24 hours.

Plasmid DNA Isolation, DNA Sequencing

The alkaline lysis method described by Sambrook et al., (Supra) was used to isolate plasmid DNA. The full length positive cDNA clone was sequenced using the dideoxy sequencing method. Sanger, et al., Proc. Natl. Acad. Sci. USA, 74:5463-5467. The open reading frame was compiled and analyzed using BLAST search (GenBank, Bethesda, MD) and sequence alignment was achieved using a BCM Search Launcher: Multiple Sequence Alignments Pattern-Induced Multiple Alignment Method (See F. Corpet, Nuc. Acids Res., 16:10881-10890, (1987). Sequences and sequence alignments are shown in FIGS. 3 through 11, 15 and 18.

Northern Blot Hybridizations of Rat Corpus Luteum RNA

Twenty μg of total RNA isolated from rat corpus luteum at various stages of apoptosis were separated on 1% denatured formaldehyde agarose gels and immobilized on nylon membranes. The full length rat apoptosis-induced eIF-5A cDNA labeled with ^{32}P-dCTP using a random primer kit (Boereinger) was used to probe the membranes (7 x 10^7 cpm). Alternatively, full length rat apoptosis-induced DHS cDNA labeled with ^{32}p-dCTP using a random primer kit (Boereinger) was used to probe the membranes(7 x 10^7 cpm). The membranes were washed once with 1x SSC, 0.1% SDS at room temperature and three times with 0.2x SSC, 0.1% SDS at 65°C. The membranes were dried and exposed to X-ray film overnight at -70°C. The results are shown in FIGS. 14 and 17. As can be seen, eIF-5A and DHS are both upregulated in apoptosing corpus luteum tissue.

Example 3

Generation of an Apoptosing Rat Corpus Luteum RT-PCR Product Using Primers Based on Yeast, Fungal and Human eIF-5A sequences

A partial-length apoptosis-induced eIF-5A sequence (SEQ ID NO. 11) corresponding to the 3' end of the gene was generated from apoptosing rat corpus luteum RNA template by RT-PCR using a pair of oligonucleotide primers designed from yeast, fungal and human eIF-5A sequences. The upstream primer used to isolate the 3'end of the rat eIF-5A gene is a 20 nucleotide degenerate primer:

5'TCSAARACHGGNAAGCAYGG 3' (SEQ ID NO. 9), wherein S is selected from C and G; R is selected from A and G; H is selected from A, T, and C; Y is selected from C and T; and N is any nucleic acid.

The downstream primer used to isolate the 3'end of the rat eIF-5A gene contains 42 nucleotides:GCGAAGCTTCCATGG<u>CTCGAG</u>TTTTTTTTTTTTTTTTTTTTT   3' (SEQ ID NO. 10). A reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out as follows:

Using 5 μg of the downstream primer, a first strand of cDNA was synthesized.

Reaction components:

RNA (5 μg)	10.5 μl
Poly(T) primer (5 μg)	1.0 μl
5X buffer	4.0 μl
dNTP(10 mM)	2.0 μl
RNase Inhibitor (4U/μl)	0.5 μl
MMLV (20U/μl)	2.0 μl

Reaction volume	20.0 μl

Reaction parameters:

37°C for 1 hour

The first strand was then used as a template in a RT-PCR using both the upstream and downstream primers.

Reaction components:

cDNA	2.00 µl
H$_2$O	32.75 µl
10X buffer	5.00 µl
MgCl$_2$(15 mM)	5.00 µl
dNTP(10 mM)	1.00 µl
Primer 1 and 2(15 µM each)	2.00 µl each
Tsg polymerase	0.25 µl

Reaction volume	50.00 µl

Reaction parameters:
94°C for 5 min
94°C for 1 min, 48°C for 1min, 72°C for 2 min, for 45 cycles
72°C for 15 min.

A partial-length apoptosis-induced eIF-5A sequence (SEQ ID NO. 15) corresponding to the 5' end of the gene and overlapping with the 3' end was generated from apoptosing rat corpus luteum RNA template by RT-PCR. The 5' primer is a 24-mer having the sequence, 5' CAGG<u>TCTAGA</u>GTTGGAATCGAAGC 3' (SEQ ID NO. 13), that was designed from human eIF-5A sequences. The 3' primer is a 30 mer having the sequence, 5' ATAT<u>CTCGAG</u>CCTTGATTGCAACAGCTGCC 3' (SEQ ID NO. 14) that was designed according to the 3' end RT-PCR fragment. A reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out as follows:

Using 5 µg of the downstream primer, a first strand of cDNA was synthesized.

Reaction components:

RNA (5 µg)	10.5 µl

Poly(T) primer (5 µg)	1.0 µl
5X buffer	4.0 µl
dNTP(10 mM)	2.0 µl
RNase Inhibitor (4U/µl)	0.5 µl
MMLV (20U/µl)	2.0 µl

Reaction volume	20.0 µl

Reaction parameters:

37°C for 1 hour

The first strand was then used as a template in a RT-PCR using both the upstream and

downstream primers.

Reaction components:

cDNA	2.00 µl
H_2O	32.75 µl
10X buffer	5.00 µl
$MgCl_2$(15 mM)	5.00 µl
dNTP(10 mM)	1.00 µl
Primer 1 and 2(15 µM each)	2.00 µl each
Tsg polymerase	0.25 µl

Reaction volume	50.00 µl

Reaction parameters:
94°C for 5 min
94°C for 1 min, 48°C for 1min, 72°C for 2 min, for 45 cycles
72°C for 15 min.

The nucleotide sequence (SEQ ID NO.1) for the full length cDNA of the rat

apoptosis-induced corpus luteum eIF-5A gene obtained by RT-PCR and the

corresponding derived amino acid sequence (SEQ ID NO.9) are depicted in FIG. 5.

Generation of an Apoptosing Rat Corpus Luteum RT-PCR Product Using Primers

Based on a Human DHS Sequence

A partial-length apoptosis-induced DHS sequence (SEQ ID NO. 6) corresponding

to the 3' end of the gene was generated from apoptosing rat corpus luteum RNA

template by RT-PCR using a pair of oligonucleotide primers designed from a human

DHS sequence. The 5' primer is a 20-mer having the sequence, 5'

GTCTGTGTATTATTGGGCCC 3' (SEQ ID NO. 17).; the 3' primer is a 42- mer

having the sequence,

5'GCGAAGCTTCCATGG<u>CTCGAG</u>TTTTTTTTTTTTTTTTTTTT 3'

(SEQ ID NO. 18).   A reverse transcriptase-polymerase chain reaction (RT-PCR) was

carried out as follows:

Using 5 µg of the downstream primer, a first strand of cDNA was synthesized.

<u>Reaction components:</u>

RNA (5 µg)	10.5 µl
Poly(T) primer (5 µg)	1.0 µl
5X buffer	4.0 µl
dNTP(10 mM)	2.0 µl
RNase Inhibitor (4U/µl)	0.5 µl
MMLV (20U/µl)	2.0 µl
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Reaction volume	20.0 µl

<u>Reaction parameters:</u>

37°C for 1 hour

The first strand was then used as a template in a RT-PCR using both the upstream and

downstream primers.

<u>Reaction components:</u>

cDNA	2.00 µl
H₂O	32.75 µl
10X buffer	5.00 µl
MgCl₂(15 mM)	5.00 µl
dNTP(10 mM)	1.00 µl
Primer 1 and 2(15 µM each)	2.00 µl each
Tsg polymerase	0.25 µl
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Reaction volume   50.00 µl

<u>Reaction parameters:</u>
94°C for 5 min
94°C for 1 min, 48°C for 1min, 72°C for 2 min, for 45 cycles
72°C for 15 min.

The nucleotide sequence (SEQ ID NO. 6) for the partial length cDNA of the rat

apoptosis-induced corpus luteum DHS gene obtained by RT-PCR and the corresponding

derived amino acid sequence (SEQ ID NO.7) are depicted in FIG. 15.

Example 4

Isolation of Genomic DNA and Southern Analysis

Genomic DNA for southern blotting was isolated from excised rat ovaries.

Approximately 100 mg of ovary tissue was divided into small pieces and placed into a 15

ml tube. The tissue was washed twice with 1 ml of PBS by gently shaking the tissue

suspension and then removing the PBS using a pipette. The tissue was resuspended in

2.06 ml of DNA-buffer (0.2 M Tris-HCl pH 8.0 and 0.1 mM EDTA) and 240 µl of 10 %

SDS and 100 µl of proteinase K (Boehringer Manheim; 10 mg/ml) was added. The tissue

was placed in a shaking water bath at 45 °C overnight. The following day another 100 µl

of proteinase K (10 mg/ml) was added and the tissue suspension was incubated in a

waterbath at 45 °C for an additional 4 hours. After the incubation the tissue suspension

was extracted once with an equal volume of phenol:chloroform:iso-amyl alcohol

(25:24:1) and once with an equal volume of chloroform:iso-amyl alcohol (24:1).

Following the extractions 1/10 th volume of 3 M sodium acetate (pH 5.2) and 2 volumes

of ethanol were added. A glass pipette sealed and formed into a hook using a Bunsen

burner was used to pull the DNA threads out of solution and to transfer the DNA into a

clean microcentrifuge tube. The DNA was washed once in 70 % ethanol and air-dried for 10 minutes. The DNA pellet was dissolved in 500 µl of 10 mM Tris-HCl (pH 8.0), 10 µl of RNase A (10 mg/ml) was added, and the DNA was incubated for 1 hour at 37 °C. The DNA was extracted once with phenol:chloroform:iso-amyl alcohol (25:24:1) and the DNA was precipitated by adding 1/10 th volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. The DNA was pelleted by centrifugation for 10 minutes at 13,000 X g at 4 °C. The DNA pellet was washed once in 70 % ethanol and dissolved in 200 µl 10 mM Tris-HCl (pH 8.0) by rotating the DNA at 4 °C overnight.

For Southern blot analysis, genomic DNA isolated from rat ovaries was digested with various restriction enzymes which either do not cut in the endogenous gene or cut only once. To achieve this, 10 ug genomic DNA, 20 µl 10X reaction buffer and 100 U restriction enzyme were reacted for five to six hours in a total reaction volume of 200 µl. Digested DNA was loaded onto a 0.7 % agarose gel and subjected to electrophoresis for 6 hours at 40 volts or overnight at 15 volts. After electrophoresis, the gel was depurinated for 10 minutes in 0.2 N HCl followed by two 15 minute washes in denaturing solution (0.5 M NaOH, 1.5 M NaCl) and two 15 minute washes in neutralizing buffer (1.5 M NaCl, 0.5 M Tris-HCl pH 7.4). The DNA was transferred to a nylon membrane, and the membrane was prehybridized in hybridization solution (40 % formamide, 6 X SSC, 5 X Denhart's, solution (1 X Denhart's solution is 0.02 % Ficoll, 0.02 % PVP, and 0.02 % BSA), 0.5 % SDS, and 1.5 mg of denatured salmon sperm DNA)). A 700 bp PCR fragment of the 3' UTR of rat eIF-5A cDNA (650 bp of 3' UTR and 50 bp of coding) was labeled with [α-^{32}P]-dCTP by random priming and added to the membrane at 1 X 10^6 cpm/ml. Similarly, a 606 bp PCR fragment of the rat DHS cDNA (450 bp coding

and 156 bp 3' UTR) was random prime labeled with [α-^{32}P]-dCTP and added at 1 X 10^6

cpm/ml to a second identical membrane. The blots were hybridized overnight at 42 °C

and then washed twice with 2 X SSC and 0.1 % SDS at 42 °C and twice with 1 X SSC

and 0.1 % SDS at 42 °C. The blots were then exposed to film for 3-10 days. The results

are shown in FIGS. 13 and 21.

Example 5

Inhibition of Apoptosis

Apoptosis is inhibited when corpus luteal cells are treated with spermidine, a

compound that inhibits the activation of eIF-5A by DHS. Six to nine days after

superovulation, rats were treated with 500 ug of prostaglandin F 2α subcutaneously.

Control rats were treated with an equivalent volume of saline solution. Fifteen to thirty

minutes later, the ovaries were removed from 4 to 8 superovulated rats, placed in EBSS

(Gibco) on ice, blotted dry and weighed. Connective tissue was trimmed away, and the

ovaries were minced finely with a razor blade and washed twice with EBSS 2 X.

Collagenase solution was prepared by vortexing 6.5 mg of collagenase (Sigma) in 5 ml of

EBSS. Minced tissue from 8 ovaries was added to 5 ml of collagenase in EBSS in a 50

ml Erlenmeyer flask and agitated gently by withdrawing several times into a Diamed

pipette. The flask with minced tissue was then placed in a water bath at 57° C for 20

minutes with gentle shaking under 95 % air : 5 % CO_2. Following this incubation, the

flask was placed on ice, and the suspension was transferred to a Nitex filter fitted with

Swiss Nitex Nylon Monofilament (75 μ) using a plastic transfer pipet. The filtrate was

collected into a 15 ml Falcon test tube. An aliquot (2.5 ml) of collagenase solution was

added to the minced tissue retained in the filter, and the suspension was transferred to a

50 ml Erlenmeyer flask and agitated gently using a pipette, incubated and filtered as

above. The two filtrates were combined, mixed with 27.5 ml of MEM (Sigma) and 1.5

ml of 10 mM glutamine and filtered again through Nitex. The dispersed cells in the

filtrate were sedimented in Falcon tubes by centrifugation using a clinical centrifuge for 5

minutes. All but 2 mls of the supernatant was removed with a pipette, and the

sedimented cells were gently resupended in the remaining 2 ml supernatant. The cells

were washed twice in 5 mls MEM by centrifugation and resupension as above and finally

resuspended in 30 mls of MEM with glutamine and with or without 10 mM spermidine

(Amersham Life Science), and incubated for 1 hour without shaking at 37 C under 95 %

air: 5 % CO_2. The cells were then sedimented by centrifugation as above and

resuspended in MEM with glutamine. The concentration of dispersed cells was

determined using a hemocytometer, and viability was assessed using trypan blue dye.

Aliquots of 2 to 5 X 10^5 cells were placed in 12 X 75 mm test tubes and incubated

without shaking for 5 hours at 37 C with or without 1 mM spermidine under 95 % air : 5

% CO_2. Following the incubation, the cells were sedimented by centrifugation, the

supernatant was removed, and the pelleted cells were frozen at ⁻80°C prior to DNA

isolation.

The degree of apoptosis in each sample was determined by DNA laddering and

degree of end labelling, both of which reflect nuclease activity. In brief, DNA was

isolated from the ovaries, labeled with [α-^{32}P]-dCTP using Klenow enzyme, separated on

a 1.8 % agarose gel, and exposed to film. The sample from control rats injected with

saline and incubated without spermidine in both incubation steps showed no evidence of

laddering or increased end-labeling (FIG. 1, lane 1). The sample from rats injected with

prostaglandin $F_{2\alpha}$ and incubated with 10 mM spermidine for the 1 hour incubation and

without spermidine in the 5 hour incubation (FIG. 1, lane 2) showed more end labeling

reflecting apoptosis-associated nuclease activity than the sample that was additionally

treated with 1 mM spermidine during the 5 hour incubation (FIG. 1, lane 3).

Example 6

In vivo Inhibition of Apoptosis by Spermidine

Apoptosis of the corpus luteum, as determined by DNA laddering and DNA end

labelling, is inhibited when superovulated rats are injected with spermidine prior to

exposure to prostaglandin $F_{2\alpha}$. Six days after superovulation, rats received three

subcutaneous injections of 0.333 mg of spermidine / 100 g rat body weight (for a total

dose of 1 mg/100 g rat body weight) 24 hours, 12 hours, and 2 hours prior to injection

with 500 μg of prostaglandin $F_{2\alpha}$ subcutaneously. Control rats received either no

injections (FIG. 2, lane 1), were injected three times with spermidine but received no

prostaglandin $F_{2\alpha}$ (FIG. 2, lane 2) or were injected three times with an equivalent volume

of saline prior to an injection with prostaglandin $F_{2\alpha}$ (FIG. 2, lane 3). The rats were

sacrificed and the ovaries removed either one hour and thirty five minutes or three hours

and forty-five minutes after injection with prostaglandin $F_{2\alpha}$. The degree of apoptosis was

determined by DNA end-labeling. In brief, DNA was isolated from the ovaries, labeled

with [α-[32] P]-dC'TP using Klenow enzyme, separated on a 1.8 % agarose gel, and

exposed to film. The DNA isolated from control rats which received no injections (FIG.

2, lane 1) and the DNA from rats treated with saline and prostaglandin Fα (FIG. 2, lane

3) showed a much higher degree of end-labeling reflecting apoptosis-related nuclease

activity than DNA from rats treated with either spermidine alone or spermidine followed

by prostaglandin $F_{2\alpha}$ (FIG. 2, lanes 2, 4, 5, 6, 7). In particular, endogenous treatment of

rats with spermidine inhibits apoptosis whether it is initiated *in situ* without exogenous

prostaglandin $F_{2\alpha}$ (FIG. 2, lanes 1 and 2) or by treatment with exogenous prostaglandin

$F_{2\alpha}$ (FIG. 2, lanes 3 and 4).

Example 7
Design of Antisense Oligonucleotides

Mfold version 3.1 by Zuker and Turner was used to predict the secondary

structure of the mRNA of the full-length rat eIF-5A. Oligonucleotides against accessible

regions of 14 base pairs or longer that were not base-paired and that existed at all three of

the lowest energy conformations based on a folding temperature of 37 °C were designed

(Antisense Oligo 1 and Antisense Oligo 2). An oligo spanning the translation start site

was also designed, although it was not predicted to be accessible (Antisense Oligo 3).

Antisense Oligo 1 : bp 618 to 635 of full-length sequence
(18 bp) 5' TTG AAG GGG TGA GGA AAA 3'

Antisense Oligo 2 : bp 845 to 859 of full-length sequence
(15 bp) 5' TTG AGT GGG ATA AAG 3'

Antisense Oligo 3 : spanning ATG start (-5 to + 13)
(18 bp) 5' AAT CAT CTG CCA TTT TAA 3'

For additional description of mfold version 3.1 software, see, e.g., Zuker M., et al.

"Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical

Guide In RNA Biochemistry and Biotechnology, 11-43, J. Barciszewski and B. F. C.

Clark, eds., NATO ASI Series, Kluwer Academic Publishers (1999); Mathews DH, et al.,

"Expanded Sequence Dependence of Thermodynamic Parameters Improves Prediction of

RNA Secondary Structure," J. Mol. Biol. 288: 911-40 (1999).